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Identification of the Conjugated Linoleic Acid Isomer Responsible for Inhibiting Essential Fatty Acid Metabolism and Tumor Cell Growth

By

David Won Lung Ma

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences - Medicine

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Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Identification of the Conjugated Linoleic Acid Isomer Responsible for Inhibiting Essential Fatty Acid Metabolism and Tumor Cell Growth submitted by David Won Lung Ma in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences - Medicine.

Dr. M.T. Clandinin

Dr. C.J. Field

Dr. P. Sporns

Dr. L.C.H. Wang

Dr. J.F. Angel

Date: July 27, 2001

ABSTRACT

Conjugated linoleic acid (CLA) refers to a group of geometrical and positional isomers of linoleic acid (LA) that have been shown to have beneficial health effects. Modulation of essential fatty acid metabolism by CLA may be a mechanism by which CLA inhibits tumorigenesis, however, the role of specific isomers is not known. Specific CLA isomers and their metabolites may compete and interfere with LA metabolism to AA, and reduce the synthesis of tumor promoting eicosanoids. Yet to be identified eicosanoids from CLA may also play a role in tumor growth.

Mechanistic studies in this area have been limited by methodological challenges. Availability of CLA from biological sources such as animal fat is low. Composition of commercial sources of CLA is variable and pure or enriched isomers are not available in quantities required for biological studies. Objectives of this study were (1) to develop a method to readily synthesize CLA from LA purified from safflower oil, (2) to separate and identify isomers by gas liquid chromatography, (3) to determine the level of CLA in Canadian beef and dairy foods, and (4) to develop a method to purify the major CLA isomers. These methods provide a foundation to study the effect of specific CLA isomers on essential fatty acid metabolism and eicosanoid synthesis.

Synthetic yields from safflower oil of upwards of 50% (wt/wt) CLA can be obtained, containing 45, 46.1 and 3.5% of the isomers, Δ9*c*,11*t*-, Δ10*t*,12*c*- and Δ9*t*,11*t*-18:2, respectively. Dairy and beef products contain the Δ9*c*,11*t*-18:2 isomer in a range between 1.2-6.2 mg/g fat or 0.03-81.0 mg/usual serving. Enriched mixtures of Δ9*c*,11*t*-, and Δ10*t*,12*c*-18:2 were prepared by urea crystallization. MDA-MB-231 mammary tumor

cells supplemented with an equal mixture of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2, or mixtures enriched in either Δ9*c*,11*t*- or Δ10*t*,12*c*-18:2 were observed to preferentially incorporate Δ9*c*,11*t*-18:2. Among these three CLA treatments, membrane phospholipid levels of LA tended to increase and arachidonic acid (AA) was reduced specifically by the Δ10*t*,12*c*-18:2 isomer. The Δ10*t*,12*c*-18:2 isomer inhibited conversion of LA to AA. This corresponded with reduced PGE_2 production and cell growth.

In summary, important methods of CLA analysis and preparation of enriched mixtures of CLA were developed. A greater understanding of the mechanism by which CLA inhibits tumor growth was also elucidated.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
A/M	Antibiotic-antimycotic
ATCC	American Tissue Culture Centre
BSA	Bovine serum albumin
BF_3-MeOH	Boron trifluoride in methanol
c	Cis double bond
CLA	Conjugated linoleic acid
^{13}C-NMR	Carbon nuclear magnetic resonance
ddH_2O	Double deionized water
DMBA	Dimethylbenz(a)anthracene
DMSO	Dimethylsulfoxide
DPM	Decays per minute
FBS	Fetal bovine serum
GLC	Gas liquid chromatography
GC-MS	Gas chromatography-mass spectrometry
HCl	Hydrochloric acid
HDL	High density lipoprotein
5, 12, and 15-HETE	5, 12, and 15 - Hydroxyeicosatetraenoic acid
HPLC	High performance liquid chromatography
^{1}H-NMR	Proton nuclear magnetic resonance
IMDM	Iscoves Modified Dulbeco Media
IR	Infrared
KOH	Potassium hydroxide
LA	Linoleic acid
LDL	Low density lipoprotein
LTB_4	Leukotriene B_4
NaOH-MeOH	Sodium hydroxide in methanol
NaOMe-MeOH	Sodium methoxide in methanol

PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PGE_2	Prostaglandin E_2
PI	Phosphatidylinositol
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PPAR	Peroxisome proliferator-activated receptor
PS	Phosphatidylserine
t	Trans double bond
TLC	Thin layer chromatography
TMG-MeOH	Tetramethylguanidine in methanol
TMSD	Trimethylsilyldiazomethane
UV	Ultraviolet
VLDL	Very low density lipoprotein

Chapter 1 - General Introduction

1. Introduction

CLA has been known as a milk fatty acid for many years and its reference in the literature can be found dating from the 1950's and 60's [1; 2]. Research interest into the biological effects of CLA originated from early investigative work of M.W. Pariza and colleagues [3-5]. The study of mutagenic and carcinogenic compounds in fried ground beef serendipitously resulted in the discovery of an anticancer agent, identified as CLA [6; 7]. To date, CLA has been shown to exhibit a variety of potentially beneficial health effects. A review of the literature since the identification of CLA as a carcinogenic inhibitor will provide insight into this interesting area of research. Methodologies particular to the study of CLA, a synopsis of the major biological effects, and possible mechanisms of action will be reviewed.

2. Definition of CLA and Commercial Composition

CLA refers to a group of positional and geometrical isomers of the essential fatty acid, LA. The chief distinguishing feature of CLA is the conjugated arrangement of the two double bonds contained within the molecule. Positional and geometrical isomers exist for cis and trans arrangements of the two conjugated double bonds along various positions of the carbon chain. This is in contrast to LA, which contains two fixed double bonds at the Δ9 and Δ12 carbon positions separated by a methylene group.

CLA was initially identified as a set of 9 isomers by Ha et al. [7] from alkali isomerized LA. This synthetic mixture included all combinations of cis and trans isomers of Δ9,11- and Δ10,12- and a single isomer of Δ11,13-18:2. Today CLA includes a wider range of isomers possibly including all cis and trans geometrical isomers between Δ7,9- and Δ12,14-18:2 [8-10]. Typically, the major isomers found in synthetic mixtures are Δ9*c*,11*t*-, and Δ10*t*,12*c*-18:2, but content is highly variable depending on the manufacturer [8; 11].

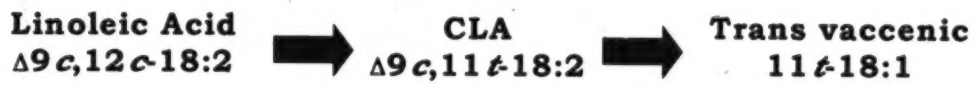
3. Dietary Sources of CLA, its Consumption and Incorporation

CLA is produced naturally in ruminant animals such as cattle, sheep and goats, hence most North Americans consume CLA, in small amounts. Through normal lipid metabolism rumen microorganisms convert polyunsaturated fatty acids such as linolenic acid and LA to monoenoic and saturated products in a process known as biohydrogenation.

A. Biohydrogenation

The ruminant microorganism, *Butyrivibrio fibrisolvens*, is responsible for the synthesis of the Δ9*c*,11*t*-18:2 isomer as an intermediate in the anaerobic biohydrogenation of LA to trans vaccenic acid (Figure 1-1) [12-18]. The two enzymes in this two step pathway have been isolated and identified and exhibit substrate specificity for LA, and the intermediate, Δ9*c*,11*t* -18:2 [17-19].

Figure 1-1 Biohydrogenation of LA and Synthesis of CLA



Chin et al. [20] demonstrate that rats are able to synthesize Δ9,11- and Δ10,12-18:2 isomers of CLA. Free LA was converted to CLA, but not esterified LA, and control rats without gut microflora were unable to synthesize CLA. Therefore, suggesting that rat microflora are capable of synthesizing CLA and unesterified LA is the substrate pool for the biosynthesis of CLA. There is no evidence to suggest human microflora can produce CLA. In humans it has been shown that esterified LA is not converted to CLA [21]. Additionally, human gut bacteria inoculated into germ free rats and fed a diet supplemented with LA as sunflower oil do not accumulate CLA in body tissues [22]. Although human microflora are unable to synthesize CLA, some evidence suggests that synthesis of CLA, in particular the Δ9*c*,11*t*-18:2 isomer may be synthesized in humans

via Δ9 desaturation from dietary trans vaccenic acid [23]. Subjects fed a high trans fatty acid diet had elevated amounts of circulating CLA [24] and when human subjects were fed deuterium labeled vaccenic acid, some of the label was recovered as deuterated Δ9*c*,11*t*-18:2 [25].

B. CLA in Foods

The presence of CLA in milk fat had already been established in the mid 1900's [1; 26-28]. The recognition of CLA as an important dietary lipid by Pariza et al. [6] prompted many studies to evaluate the content of CLA in dairy and beef products. These studies have been conducted in many countries such as, Australia, the United States and in several European countries [29-39]. All studies have identified Δ9*c*,11*t*-18:2 as the major CLA isomer in these foods comprising greater than 80-90% of total CLA content. In contrast to foods, synthetic mixtures contain several other isomers of CLA [7; 8; 11].

Analysis of CLA content in Canadian foods by our laboratory has shown that CLA is a minor component of dietary fat in ruminant products such as dairy and beef products [40]. This data is presented in Chapter 5 of this thesis. Typically, levels of CLA in products from ruminant animals were found to range between 1.2 - 6.1 mg/g fat.

A growing body of research has been devoted to developing approaches to enhance CLA content in milk and muscle of ruminants such as dairy cows [41], steers [42], lamb [43] and goats [44]. There is also interest by food scientists in increasing CLA content in processed dairy products [45], cheese [46], and butter oil [47]. Incorporation of CLA into non-ruminant animal products such as chicken eggs [48-51], pigs [52] and even striped bass [53] have been explored. Sensory evaluation of animal meats from pork and cattle enriched in CLA exhibit no difference in physical properties or quality [54; 55]. The oxidative stability of CLA in food is most likely no different relative to other polyunsaturated fatty acids [36; 46]. However, oxidative studies of the isolated fatty acid has shown it to be both less oxidized relative to LA [6] and more readily oxidized [56; 57]. The relevance of this fact is of importance chiefly for encapsulated CLA supplements and not for CLA found in food.

C. CLA Intake

Current levels of CLA intake are estimated to be less than 1 g/day. Fritsche and Steinhart [58] estimated that daily CLA intake in German men was approximately 430 mg and 350 mg in women. This estimate was based on the typical German food basket, not on actual food consumption and also assumes that all food purchased is eaten. CLA intake has not been previously documented from food intake records in free-living subjects.

Our laboratory has estimated intake of Δ9*c*,11*t*-18:2 based on diet records for a group of young Canadian adults using food compositional data determined in Chapter 5 of this thesis. Intake of Δ9*c*,11*t*-18:2 was estimated from 22 free-living Canadians by analyzing two 7-day diet records taken six months apart [59]. Mean CLA intake was determined to be 94.9 ± 40.6 mg/day with intakes ranging between 15-174 mg/day [60]. Consumption of the Δ9*c*,11*t*-18:2 isomer from actual 7-day food records in this study is much lower than that reported for total CLA intake estimated by Fritsche and Steinhart [58].

In rats the minimum amount of CLA associated with anti-carcinogenic effects is 0.1% (w/w of diet), and Ip et al. [61] extrapolated this level on a weight basis to 3 g/day in humans. On an energy basis the rat diet represents approximately 300 mg CLA/42000kJ or per 1000 kcal. Extrapolating the 300 mg/1000 kcal value to the human diet (2000 kcal/day) corresponds to approximately 600 mg CLA/day, considerably less than the original estimate of 3 g/day. A level of 600 mg/day is more attainable given that actual intake is 94.9 mg/day and estimated intake can be as high as 350 – 430 mg/day. Studies are still required to determine the efficacious level of CLA in humans.

D. Tissue Incorporation of CLA

Understanding which tissues accumulate CLA may provide insights into its biological function. CLA has been detected in various human tissues [62-70] and levels of CLA can be modulated by dietary intervention [29; 32; 68; 70-72]. Typical levels of CLA found in human plasma falls in the μM range and investigators have reported levels

of 7.1 μM [21; 72] and 10-40 μM [73]. In a study following typical eating patterns of 123 Swedish men, CLA content in adipose and serum was found to represent 0.50 and 0.25% (w/w) of total fatty acids, respectively and CLA was positively correlated with the proportion of milk fat to fat intake [70]. CLA has also been found in human breast milk and most likely derived from the mothers consumption of dairy foods [29; 38; 71; 74-76].

CLA incorporation into tissues has been more extensively examined in animal studies [52; 77]. In a comprehensive assessment, Sugano et al. [77] found that in rats fed 1% (w/w) CLA over 2 weeks, both uptake and isomeric distribution in brain, heart, liver, serum, spleen, kidney, lung and adipose tissues varied markedly. Adipose tissues incorporated the greatest amount of CLA, ~9% of fatty acids. Brain tissues incorporated the least amount of CLA, <1% of fatty acids, and only Δ9*c*,11*t*-18:2 was incorporated [77]. CLA is also found in expressed breast milk in rats [78].

It is evident that CLA in the diet can be readily obtained from a variety of food sources and once consumed is incorporated into biological tissues. To date, studies have mainly used mixtures of CLA isomers. The focus of current research is to understand the biological role of specific CLA isomers and their mechanism of action(s), especially the Δ9*c*,11*t*-18:2 isomer, which is found in greatest natural abundance. Before much of the investigative work in this area could begin, several methodological hurdles need to be overcome.

4. Analysis of CLA

Early methodological hurdles in this area of research pertained to the preparation, separation, identification and quantification of CLA isomers.

A. Synthesis of CLA

The common method used to synthesize CLA involves alkali treatment of LA. Alkali isomerization of LA involves the shifting of either the double bond at the Δ9 or Δ12 position such that a more stable conjugated double bond system is produced [79]. Positional isomers of Δ9,11- and Δ10,12-18:2 are most favored by this reaction scheme.

Geometrical consideration of cis and trans positions of the bonds limits the total number of possible outcomes. If all possible cis and trans combinations occur, then there are eight such possibilities, Δ9*c*,11*c*-, Δ9*t*,11*t*-, Δ9*c*,11*t*-, Δ9*t*,11*c*-, Δ10*c*,12*c*-, Δ10*t*,12*t*-, Δ10*c*,12*t*- and Δ10*t*,12*c*-18:2. Only one double bond shifts in the isomerization, and assuming there is retention of configuration of the stationary bond, this restricts the total number of possible outcomes. LA has two cis double bonds at the Δ9 and Δ12 positions, then Δ9*c*,11*c*-, Δ9*c*,11*t*-, Δ10*c*,12*c*- and Δ10*t*,12*c*-18:2 are the only possible outcomes. Alkali isomerization involves a prototrophic shift mechanism [79]. Initially, hydrogen is abstracted from the methylene bond, thus producing an anion at the Δ11 position. This anion has three resonance structures, which forces the carbons into a coplanar configuration. Not all the coplanar configurations are possible due to steric effects, thus restricting the number of possible isomers. These positional and geometrical considerations theoretically limit the alkali isomerization of LA to two outcomes of, Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 [79]. These two isomers are typically the major isomers in synthetic mixtures [6; 7]. If the reaction process is carried out under high temperature and/or prolonged time this results in the formation of additional positional and geometrical isomers.

A biological system employing the use of enzymes from *Butyrivibrio fibrisolvens* is another possible route for synthesis of CLA [16-18]. The overall system involves the biohydrogenation of LA to a monoene, 11*t*-18:1, vaccenic acid. The enzyme responsible for the first step is linoleate 12cis,11trans isomerase [17]. This step highly favors the synthesis of the Δ9*c*,11*t*-18:2 isomer of CLA. The second step involves the conversion of the Δ9*c*,11*t*-18:2 intermediate to a trans monoene via the enzyme, cis9,trans11-octadecadienoate reductase [19]. The synthesis of CLA by culturing *Butyrivibrio fibrisolvens* has not been used extensively but has been described by Chin et al. [33].

A third method of synthesizing CLA involves the dehydration of ricinoleic acid. Ricinoleic acid can be thought of as oleic acid (Δ9c-18:1) with an alcohol attached at the Δ12 position. An inexpensive source of ricinoleic acid is castor oil. The dehydration of ricinoleic acid is simply the dehydration of an alcohol, producing a double bond. Dehydration is carried out in acidic conditions to convert the hydroxyl group into a good

leaving group. The reaction proceeds via an E1, unimolecular elimination mechanism, such that the most highly substituted product is produced. The new double bond may form at either the Δ11 or Δ12 positions by the "Saytzeff rule" [80]. The double bond at the Δ9 position will help favor the formation of the new double bond at the Δ11 position owing to the greater stability conferred by conjugated systems. Body et al. [81] report a yield of 33.7% total CLA by this method consisting primarily of 20.3% cis,trans/trans,cis, 8.0% cis,cis and 5.4% trans,trans isomers. A recent review of this method has been described by Berdeaux et al. [82].

Another useful method to consider is the synthesis of deuterium labeled Δ9*c*,11*t*-18:2,-9,10d_2, which is described by Adlof [83]. Methyl santalbate is reduced with Lindlar catalyst and deuterium to rapidly prepare labeled CLA in gram quantities. This method could also be used specifically to synthesize the Δ9*c*,11*t*-18:2 isomer.

Large-scale preparation of CLA on a gram to kilogram basis is required to conduct animal, and human trials. Each method described previously has both advantages and disadvantages. The alkali isomerization of LA is a simple approach that produces CLA in large quantities but also produces a large number of minor isomers. The enzymatic approach produces a single isomer, Δ9*c*,11*t*-18:2, but in limited quantities. The dehydration of ricinoleic acid favors the synthesis of Δ9,11 isomers, but further isomerization may also occur. To date, alkali isomerization of LA is the common method employed by many investigators [7; 33; 84; 85].

B. Enrichment of CLA Isomers from Mixtures

The production of large quantities of mixtures of CLA isomers can be readily accomplished. However, the production of single isomers is not as easily accomplished. The approach most often employed is the isolation or enrichment of isomers from mixtures. The enrichment of specific CLA isomers from complex mixtures has been accomplished on a limited scale using methods of low temperature crystallization, and lipase catalyzed esterification.

Low temperature crystallization of a mixture of CLA isomers in an organic solvent will result in selective enrichment of a particular isomer. Multiple crystallizations

in various solvents may be required depending on the approach. Low yield of product is typical of this procedure [79; 86].

The use of lipases to selectively fractionate CLA has been shown feasible using a lipase from *Geotrichum candidum* [87; 88]. From a mixture of free fatty acids Δ9*c*,11*t*-18:2 was selectively esterified. The unreacted free fatty acid component was subsequently enriched in Δ10*t*,12*c*-18:2. The level of enrichment of these major isomers were relatively high at >80%. The process of selective enrichment using lipases can be further utilized to incorporate CLA isomers into triglycerides. Currently, it is common practice to add CLA as free fatty acids into animal diets and cell cultures. Feeding triglycerides containing CLA would present a more physiological process of intake. Garcia et al. [89] have used the lipase, Chirazyme L-2 from *Candida antarctica* to incorporate CLA into butteroil triglyceride. Martinez et al. [90] have also used this approach to incorporate CLA into corn oil triglycerides using both Chirazyme L-2 from *Candida antarctica* and IM-60 from *Mucor miehei*. McNeil et al. [87] have used *Rhizomucor miehei* to esterify CLA to palm oil triglycerides.

Development of additional purification methods will most likely continue as a recent approach has been described for the use of supercritical fluid processing to concentrate CLA from milk [91]. Although the methods described can yield purified CLA isomers, the scale of production and associated costs restricts their usefulness primarily for conducting cell culture and perhaps small animal studies. The ultimate goal is to develop a simple approach that can be scaled for large production of enriched isomers for use in large animal and human studies.

C. Extraction, Saponification and Methylation

An early method describing the isolation and separation of CLA from food and tissue was described by Ha et al. [6]. In general, lipids are extracted from biological materials using organic solvents. Extracted fats and oils are then saponified to release free fatty acids. The process of extraction and saponification do not produce CLA as shown by Ha et al. [7]. CLA is then separated from all other fatty acids by reverse phase-high

performance liquid chromatography (HPLC) and then resolved by gas liquid chromatography (GLC).

Saponification and methylation steps are necessary for their respective roles in the process of releasing fatty acids from fats and oils, and methyl derivatization for GLC analysis. Werner et al. [35], Chin et al. [33], and Shantha et al. [92] show that CLA is susceptible to intraisomerization and interisomerization. The matter of intraisomerization is of greatest relevance in accurately characterizing endogenous amounts of CLA from foods and tissues. Temperature and/or duration can affect the normal distribution of CLA by methylating with 14% boron trifluoride in methanol (BF_3-MeOH) [35]. Even short treatment periods of 0.5 min at 100°C or 15.0 min at 50°C result in a change in CLA profile. However, methylating with 14% BF_3-MeOH for 30 min at 20°C were ideal conditions that did not alter the distribution of CLA nor create unidentifiable peaks as identified by GLC analysis [35]. Longer treatment periods of 120 min at 20°C did not alter the distribution of CLA. It appears that heating is a factor, which causes intraisomerization of CLA.

Shantha et al. [92] compared four methods to transesterify CLA. Methods were evaluated to determine if they resulted in intraisomerization of CLA. The four methods evaluated were; sodium methoxide in methanol (NaOMe-MeOH), tetramethylguanidine in methanol (TMG-MeOH), American Oil Chemists' Society (AOCS, Ce2-66) method, which uses sodium hydroxide in methanol (NaOH-MeOH), and BF_3-MeOH, and direct transesterification with methanol-benzeneacetyl chloride. Overall, the NaOMe-MeOH and TMG-MeOH methods were shown not to cause intraisomerization. Shantha determined that the AOCS method causes intraisomerization which results in 3% reduction in Δ9*c*,11*t*-18:2 abundance, which confirms the earlier observations made by Werner et al. [35]. Therefore, investigators have shown the need for careful selection of reaction conditions best suited for the analysis of CLA.

D. Chromatographic Separation and Identification

The identification and quantification of CLA isomers is commonly done by capillary GLC analysis and is the simplest method able to discriminate between different

geometrical and positional isomers of CLA. Initial GLC analysis by Ha et al. [7] identified 7 peaks by GLC containing 9 isomers of CLA. The isomer of interest, Δ9*c*,11*t*-18:2 coeluted with the Δ9*t*,11*c*-18:2 isomer. The number and variation of CLA isomers presents technical challenges in the analytical analysis of CLA. Early analyses were plagued by poor resolution of the many isomers present in mixtures of CLA. This has hindered the identification of minor isomers and quantification of isomers of interest.

Argentation HPLC of CLA provides very good separation on the basis of the geometry of the double bonds. Resolution and separation is improved over GLC using multiple columns linked together in series [9]. Long chain metabolites of CLA have also been successfully separated and identified using argentation HPLC [93]. Typically, CLA is separated as its fatty acid or methyl ester. Conversion of CLA to p-methoxy derivatives permits similar separation without the need for multiple columns [94]. It should be noted that one advantage of HPLC over GLC is the recovery of product for further derivatization or fractionation purposes. But, GLC provides greater sensitivity and greater reproducibility of retention times.

A variety of other techniques have also been employed in the analysis of CLA including carbon nuclear magnetic resonance (^{13}C-NMR) [95], proton nuclear magnetic resonance (^{1}H-NMR) [6; 96; 97], infrared (IR) [98-100], and ultraviolet spectroscopy (UV) [7; 100]. GLC and argentation HPLC remain the preferred methods for the analysis of CLA for their ability to separate CLA isomers well and their ease of use.

5. Biological Effects

Although the mechanism of action is unknown, the biological effects of CLA are evident in many animal and cell culture models. These effects can be categorized into several main topics associated with anti-carcinogenicity, anti-atherogenicity and nutrient metabolism, which has been summarized in greater detail in Appendix A.

A. Anti-carcinogenic Effects

The initial observation that CLA was able to inhibit the incidence of chemically induced tumors has lead to a great number of studies supporting this effect in a variety of

animal and cell-line models [3; 6; 61; 84; 101-103]. These experimental models have excluded effects that may be attributed to the source or form of CLA. Both natural and synthetic CLA were shown to be effective at inhibiting tumor growth. Purified extracts of CLA from fried ground beef or synthetic mixtures were both shown to inhibit carcinogenesis [3; 6]. CLA as either the free acid or esterified in triglyceride exhibit similar effects [104]. This observation is important because the free acid form of CLA is currently more available.

To date, animal and in-vitro models have shown that feeding a mixture of CLA can inhibit tumorigenesis in skin, forestomach, colon, and mammary tissues [3; 6; 84; 101; 105-107]. Several investigators using human cell culture models have observed cytostatic and cytotoxic effects of CLA. In human cell lines of colorectal (HT-29), melanoma (M21-HPB) and breast cancer (MCF-7), CLA reduced the growth of these cancer cells and had the most impact on breast cancer [105; 106]. Physiological levels of CLA ($x10^{-5}M$) resulted in inhibition of cell growth of colonic and skin cancers and had cytotoxic effects on breast tumor cells [105]. Colonic and skin tumor cells exhibited both dose and time dependent response to CLA administration and coincided with decreased nucleic acid uptake [106].

A cautionary note should be made about the design of cell culture experiments involving LA and CLA. LA is an essential fatty acid required in the diet. It may not be appropriate to compare LA directly to CLA, which may only be a comparison of an essential fatty acid adequate versus inadequate state. Any treatment with CLA should also include LA and lack thereof may alter the response of cells towards CLA.

The anti-carcinogenic effect is most responsive in the breast cancer model and the focus of many studies. Ip et al. [84] show that CLA can inhibit 7,12-dimethylbenz(a)anthracene (DMBA) induced mammary tumorigenesis in a dose dependent manner, with maximal protection achieved at a dose of 1.0% (w/w) of diet. Levels of CLA intake greater than 1% of diet resulted in accumulation in neutral lipid [108]. Inhibition of tumor growth was achieved through oral intake of CLA, versus topical application in earlier experiments [3; 6; 101].

When CLA is consumed during post-weaning and into early puberty at levels of 0.5% and 1% (w/w), mammary tumor incidence is reduced at all stages of carcinogenesis from initiation, promotion to progression, [104]. Notably, consumption of CLA by mice prior to injection of cancer inducing agents conferred long lasting protection [104; 109]. Alternatively, continual intake of CLA is required if the carcinogen administration precedes CLA intake to confer protection. In this instance CLA is not protective against chemically induced mammary cancer in the short term (4 or 8 weeks), and benefits are realized only after prolonged intake of CLA (20 weeks) [108]. Contrary to this, in a transplantable murine mammary tumor model, short term feeding for 45 days with CLA did not inhibit tumor growth [85]. Overall, the published research suggests that short-term feeding of CLA after the onset of carcinogenesis in either the inducible or transplantable mammary models is not beneficial. This suggests that CLA is not a strong therapeutic agent but rather a preventative agent of cancer.

CLA has been shown to inhibit the formation and increase the latency of tumors. CLA has also been shown to inhibit metastasis in SCID mice transplanted with human prostate tumor cells (DU-145) [110] and in mice transplanted with the mouse mammary tumor cell line 4526 [111]. As CLA increased from 0 to 1% in diet Hubbard et al. [111] show that the effects of CLA were similar to that in animals treated with indomethacin, a prostaglandin inhibitor. This suggests that CLA alters prostaglandin E_2 (PGE_2) production or has effects on the PGE_2 receptor.

Indirect evidence suggests that CLA maybe a beneficial component in human diets. In a large study of 4697 women, initially cancer free, Knekt et al. [112] have demonstrated that women consuming higher levels of dairy products have decreased incidence of breast cancer.

B. Anti-atherogenic Effects

Two early studies demonstrated a positive role for CLA in reducing the risk of atherosclerosis in hamster and rabbit models [113; 114]. Lee et al. [113] fed rabbits a diet containing 14% fat and 0.1% cholesterol with or without 0.5 g CLA/day over a 22 week period resulting in a decrease in plasma low density lipoprotein (LDL) cholesterol by

week 8, and high density lipoprotein (HDL) levels were unchanged. Triglyceride levels and fatty streak lesion size were reduced in CLA fed animals compared to controls. Nicolosi et al. [114] observed in the hamster model over an 11 week period that feeding as little as 0.06% CLA as energy resulted in significant decrease in total cholesterol, LDL cholesterol, and triglyceride, but HDL cholesterol remained unchanged. Morphologically, fatty streak formation of the aorta was reduced in CLA fed animals. In both animal models, CLA exhibited a hypocholesterolemic, lipid reducing effect and a reduction in fatty streak formation. These studies have provided some interesting findings, however more work is still needed to identify the yet to be determined mechanism of action.

In contrast to the results shown by Lee et al. [113] and Nicolosi et al. [114], Munday et al. [115] have shown that CLA promotes fatty streak formation in the C57BL/6 mouse model of atherosclerosis. Despite achieving a favorable lipid profile in which CLA treated mice showed higher HDL-cholesterol to total cholesterol ratio and lower triglyceride than controls, these animals had increased fatty streak formation. It was speculated by the authors that fatty acid streak formation induced by CLA occurred independent of the improvements in lipid profile and was the result of immune stimulation of foam cells leading to the deposition of lipids at the lesion.

Some work has examined the effects of specific CLA isomers. Yotsumoto et al. [116] have shown that Δ10*t*,12*c*-18:2 inhibits triglyceride and cholesterol ester synthesis and apoB secretion relative to the Δ9*c*,11*c*-18:2 in HepG2 cells. No data was available on the effects of the Δ9*c*,11*t*-18:2 isomer. A similar effect was reported by De Deckere et al. [117] showing that in hamsters Δ10*t*,12*c*-18:2 decreased total plasma cholesterol, increased very low density lipoprotein (VLDL) cholesterol and decreased LDL and HDL-cholesterol, and increased plasma triglyceride. There was no reported effect of the Δ9*c*,11*t*-18:2 isomer on any of these parameters. Using the Syrian Golden Hamster model, Gavino et al. [118] showed that a mixture of CLA isomers decreased plasma triglyceride but not LA or Δ9*c*,11*t*-18:2. In this study, the level of the Δ9*c*,11*t*-18:2 isomer in the mixture and in the single isomer preparation were similar, but the total amount of CLA was different in the diets prepared. Thus, synergistic effects among isomers can not be discounted.

C. Nutrient Metabolism

CLA-fed animals exhibit changes in body composition, energy metabolism, food intake and weight gain and most profoundly by decreasing body fat and increasing lean tissue mass [78; 119-121]. CLA fed animals show improved feed efficiency [54; 78; 119; 122] and reduced cachexic like wasting in vaccinated animals [123], which has many practical applications for animal production. An early study by Park et al. [120] show that CLA fed mice exhibited no change in body weight, however body compositional analysis showed 57-60% reduction in body fat and 5-14% increase in lean tissue mass compared to controls. In fat pad and skeletal muscle, it was observed that CLA increased the activity of carnitine palmitoyltransferase, and decreased the activity of lipoprotein lipase in cultured 3T3 L1 adipocytes [120]. Therefore, it appears that CLA enhances lipid oxidation and causes a reduction in fat storage possibly by affecting the activity of key enzymes of lipid metabolism.

There is some evidence suggesting that CLA does not alter nutrient metabolism. Stangl et al. [124] report that when feed intake is controlled in Sprague Dawley rats and CLA content in the diet is varied from 1, 3 or 5% (w/w) there is no effect on body weight or partitioning of fat and protein. Some improvements in lipid profile were reported with a reduction in LDL-cholesterol, HDL cholesterol, but only at 3 and 5% CLA intake. However, translation of these levels of CLA to the human diet would be extremely high. In a similar study done in pigs fed isocaloric diets, no change in fat or lean tissue partitioning, or energy expenditure was observed in either growing or adult pigs [125; 126].

It appears that metabolic effects of weight reduction and partitioning of lean and fat mass is dependent on feeding protocols (ad lib versus restricted) as well as the type of animal model. Further studies are definitely warranted to clarify these effects.

The beneficial effects of CLA on body metabolism have potential therapeutic use for diabetics. Houseknecht et al. [127] show that CLA can normalize glycemic control in diabetic Zucker rats. This effect may be the result of CLA activation of peroxisome proliferator-activated receptor (PPAR) γ, which has been shown in adipocyte cultured cells [127]. The effects of CLA in this model mirrored effects similar to troglitazone type

diabetic drugs and therefore may provide a dietary means for treating diabetes [127]. There is some recent work showing that CLA may reduce appetite in rats [128]. A major regulator of appetite is leptin and Medina et al. [129] have shown in adult women that CLA reduces circulating leptin, however, appetite was unchanged over the 64 day study.

There are also additional areas of research showing that CLA may impact on other conditions such as Lupus [130] and may have anti-microbial properties [131; 132]. As interest in CLA expands into new areas, research must also be directed at gathering epidemiological data to substantiate biological effects of CLA observed in animal and cell culture studies.

6. Mechanism of Action

The mechanism of CLA action is an area of active investigation. It is unlikely that CLA mediates its many different effects via a single mechanism. It is not known if these mechanisms act in an independent manner or synergistically. The effect of individual CLA isomers is also not well known, which further adds to the complexity of trying to determine its mechanism of action. Possible mechanisms are summarized in Table 4 with corresponding references. The major areas will be discussed further.

A. CLA as an Antioxidant

Ha et al. [101] very early on suggested that CLA is oxidized to an iron chelator by the formation of a β-hydroxy acrolein moiety, based on UV absorption profiles for CLA after air oxidation. Oxidized CLA in tissue cell membranes could then potentially act as an antioxidant [101]. Ip et al. [84] observed a decrease in thiobarbituric acid-reactive products in the rat mammary gland, when CLA was fed. However, no decrease in lipid peroxide products in the liver was observed, as compared to positive control groups, which were treated with vitamin E or butylated hydroxyanisole. It was noted that only 0.25% CLA (w/w) in the diet was required to maximally decrease thiobarbituric acid-reactive products, but 1.0% CLA was required for maximal tumor suppression. The authors suggested that other mechanisms could be cooperatively involved with CLA to inhibit tumorigenesis.

Table 1-1 Reported Mechanisms of CLA Action

Exerts antioxidant properties against free radical damage	[84; 101; 133-140]
Induces apoptosis in certain cell types	[141-146]
Alters cell cycle distribution of cells	[141; 147; 148]
Inhibits eicosanoid synthesis	[77; 102; 149-160]
Directs gene regulation:	[117; 127; 161-165]
Modifies immune function of cells and levels of secretory products	[85; 119; 120; 146; 151; 158; 160; 166; 167]
Elongation & desaturation of CLA and competition with n-6 fatty acids (linoleate and arachidonate) into membrane phospholipid	[93; 101; 102; 117; 121; 124; 137; 145; 155; 158; 167-177]
Changes leptin and appetite	[128; 129; 146]
Alteration in lipid profile of TG, lipoproteins, cholesterol	[77; 113-118; 124; 159; 177-184]
Exerts prooxidant properties in biomembranes	[85; 135; 149; 170; 185-189]
Alters protein/enzyme function	[110; 117; 120; 152; 154; 173; 183; 184; 190-194]
Alters signal transduction	[108; 143; 148; 150; 152]
Changes tissue morphology	[145; 146; 168; 195; 196]

Other oxidized products derived from CLA may have potential antioxidant roles. Yurawecz et al. [139] show that neat and methyl esters of CLA can be oxidized to form furan fatty acids, which have known antioxidant properties. Four furan fatty acids 8,11-epoxy-8,10-octadecadienoic, 9,12-epoxy-9,11-octadecadienoic, 10,13-epoxy-10,12-octadecadienoic, and 11,14-epoxy-11,13-octadecadienoic were derived from CLA [139]. It is not yet known if these products have biological activity.

The other argument is that CLA does not have any antioxidant properties. Van den Berg et al. [134] show that CLA is more susceptible to oxidation relative to LA and biomembranes composed of 1-palmitoyl-2-linoleolyl phosphatidylcholine subjected to oxidative stresses could not be protected by CLA. This model system suggests that CLA may not be acting as an antioxidant at the level of the membrane. Others have also shown that CLA is much more readily oxidized than LA [57] and thus potentially acts as a prooxidant [187]. In a study of 53 subjects consuming 4.2 g of CLA/day, CLA enhanced both free radical and enzymatic lipid peroxidation using 8-iso-$PGF_{2\alpha}$ and 15-keto-

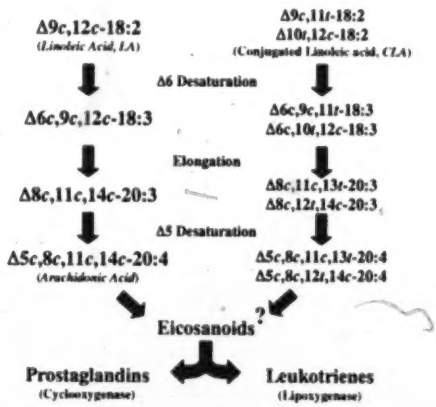
dihydro-$PGF_{2\alpha}$ as markers of these processes [186]. In some reports, cytotoxicity of cancer cells by CLA treatment has been associated with increased lipid peroxidation [136; 188].

A recent report suggests that CLA may increase vitamin A status [197]. Vitamin A also has antitumor effects and may play a role in inhibiting LA metabolism by lipoxygenase and this may be a mechanism by which CLA modulates eicosanoid synthesis [197].

B. Competition with Essential Fatty Acids

The major isomer detected in animal tissues is the Δ9*c*,11*t*-18:2 isomer, found primarily in the phospholipid fraction [29; 33; 84; 101]. CLA has been identified in different phospholipid classes [102; 124] showing selective incorporation of the Δ9*c*,11*t*-18:2 isomer [77]. Redistribution of phospholipid classes may also occur, resulting in an increase in phosphatidylcholine and a decrease in lysophosphatidylethanolamine and ethanolamine plasmalogen [124]. Therefore, detection of CLA in this fraction is suggestive of both structural and functional roles. However, effects of individual isomers have yet to be determined.

Figure 1-2 Essential Fatty Acid Metabolism and Eicosanoid Synthesis



Illustrated are potential sites of interaction and competition for elongase and desaturases. Eicosanoids derived from CLA have not yet been identified.

Ha et al. [6] initially hypothesized that CLA may compete with LA as substrate in the synthesis of AA which is the precursor of prostaglandins and leukotrienes (Figure 1-2). Competition may occur primarily by displacement of LA from membrane phospholipid in the liver [121; 124] but this does not appear to be the same in mammary tissue. The distribution of LA and AA in total mammary phospholipid remained unchanged in rats fed 1% (w/w) CLA with either a high (12% w/w) or low (2% w/w) content of LA in the diet [169]. Therefore, it would appear that CLA exerts its effects independent of LA in the diet. Although there was no change in LA or AA distribution in total phospholipid, there may be changes within the individual phospholipid classes, which were not assessed.

Sugano et al. [158] have shown that there is a trend towards reducing LA but not significantly in spleen lymphocytes, but does significantly reduce arachidonic acid (AA) content in total lipid of spleen lymphocytes in rats fed CLA. A similar observation was made by Banni et al. [168] showing that CLA did not reduce LA, but significantly reduced 18:3(6), 20:3(6), and 20:4(6), elongated, desaturated forms of LA in rat mammary tissue.

CLA can be desaturated and elongated [121; 170; 171; 175] (Figure 1-2). These products may have important biological effects in competing or inhibiting the actions of eicosanoid derived prostaglandins such as PGE_2 [77; 102; 120; 149] (Figure 1-2). Banni et al. [170] have detected conjugated Δ18:3 and Δ20:3 fatty acids in rat livers, which were non-diet derived. Sebedio et al. [93; 175] identified and characterized long chain polyunsaturated fatty acids of Δ8,12,14-20:3, Δ5,8,12,14-20:4 and Δ5,8,11,13-20:4 from rats fed diets high in CLA (180 mg/day). These products are speculated to have arisen from the metabolism of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 by elongation and desaturation pathways [175]. Belury et al. [121] demonstrate in an in vitro assay that CLA can be desaturated to a Δ18:3 product by Δ6 desaturase. Alternatively, CLA may inhibit the ability of the Δ6 and Δ9 desaturases to metabolize n-6 fatty acids. Bretillon et al. [173] have shown that the Δ9*c*,11*t*-18:2 isomer inhibits Δ6 desaturation of LA and the Δ10*t*,12*c*-18:2 isomer inhibits Δ9 desaturation of stearic acid.

Although the function of these long-chain desaturated products are not known, several investigators have observed inhibitory effects of CLA on eicosanoid production in both animal and cell culture models. Release of PGE_2 is reduced in cultured keratinocytes [155], rat serum and immune cells [77; 158] and cultured bone cells [153] by incubating with CLA. Release of another potent eicosanoid, Leukotriene B_4 (LTB_4) has also been shown to be inhibited by CLA in peritoneal exudate cells [77].

C. Modulating Immune Function

Immune function is stimulated by CLA in animal and cell-culture models. An early study reported that CLA prevented growth retardation and weight loss in chicks and rats resulting from catabolic effects due to immune stimulation or endotoxin exposure [167] and was associated with increased macrophage response and phagocytosis.

Lymphocyte proliferation and increased IL-2 production has been observed in porcine cultures supplemented with CLA [85]. In young and old mice, Hayek et al. [151] show that CLA fed animals exhibited greater proliferation of splenocytes and had increased IL-2 production, but no effect on IL-1 or PGE_2 production, natural killer cell activity or delayed type hypersensitivity responses of immune cells. Chew et al. [166] observed an inhibitory effect of CLA on IL-2 production but there remained an enhancing effect on lymphocyte proliferation, lymphocyte cytotoxicity and macrophage killing ability. Other aspects of the immune system such as immunoglobulin production have also been shown to be affected by CLA. Sugano et al. [158] observed that CLA can modulate immunoglobulin levels, increasing IgA, IgG and IgM while decreasing IgE.

The immune system may not play a direct role in mediating anti-tumor effects of CLA. In a short term feeding trial using a transplantable tumor model, CLA altered immune function but did not affect changes in tumor growth [85]. In a SCID mouse model, lacking a host defense system, CLA was inhibitory to inoculated human breast adenocarcinoma cells, MDA-MB-468 [107], however CLA was not able to prevent metastasis of the engrafted cells, compared to control animals.

It is evident that CLA can modulate aspects of the immune system, however the role of CLA in modulating immune function as a mechanism of action is not clearly

understood. Future studies in this area require greater understanding of the effects of CLA in the context of diets comparable to human diets in terms of total and type of fat. Turek et al. [160] show that effects of CLA on immune function are dependent on the n-6 to n-3 fatty acid ratio in the diet.

D. Altering Gene Transcription, PPARS

The PPARs are nuclear transcription factors, which regulate expression of many enzymes of lipid metabolism such as liver fatty acid binding protein, peroxisomal acyl-CoA oxidase and microsomal cytochrome P450 [198]. CLA appears to mimic the effects of hypolipidemic agents [161], which act through PPARs. CLA has been shown to be a ligand for PPARα and PPARβ [164; 165]. The Δ9*c*,11*t*-18:2 isomer is the most potent ligand followed by Δ10*t*,12*c*-18:2 [164]. It should be noted that there are species-specific effects of CLA in various rodent models, which hinders the extrapolation of these results to humans [199]. Although CLA activates PPARs, it does not activate other transcription factors such as NF-κB [200].

7. Toxicity and Human Studies

The potential toxicity of CLA has been extensively examined by Scimeca [196]. In a 36 week trial feeding Fischer 344 rats supplemented with 1.5% (w/w) of CLA, it was concluded no toxic effects were associated with CLA consumption. CLA supplementation did not alter body weight gain, organ weights and haemotological parameters. The author did not provide any data but indicated that histopathological evaluation of the major organs showed no differences. The author noted that the level of CLA consumed by the rats was significantly greater than that typically consumed by humans, and the lack of toxicity in any of the measured parameters would indicate that CLA is safe for consumption. In a recent study, C57BL/6J mice were shown to have marked fat loss but also developed lipodystrophy consuming a semipurified diet supplemented with 1% (w/w) CLA over an 8 month period [146]. There was no difference in total body weight, however in CLA fed mice, there was a marked increase

in liver weight and significant decrease in white adipose and total loss of brown adipose tissue. CLA fed mice also exhibited insulin resistance. Both Schimeca [196] and Tsuboyama et al. [146] used similar levels of a mixture of CLA, 1.5 and 1.0 % (w/w), respectively. The disparity in results observed by may be attributed to the use of different rodent models.

The results of several human studies have generally shown that CLA in the short-term is safe for consumption [201-203]. Several studies have focused on the effect of CLA on body weight or fat mass, which has been documented from animal studies. Surprisingly very little or no effect has been observed with CLA treatment in most of the studies. Basu et al. [204] have shown that CLA increases lipid peroxidation in human (27 male, 26 females) subjects fed 4.2 g CLA/day over a 3 month period. In a separate study, Basu et al. [186] again showed that obese male subjects consuming 4.2 g CLA/day had increased lipid peroxidation after 1 month, but were otherwise healthy and not adversely affected by CLA. Berven et al. [201] conducted a study to assess the safety of CLA in a 12 week study with 55 subjects consuming 3.4 g CLA/day. Blood analysis and clinical assessment of the subjects did not reveal any adverse effects, but there were reports of adverse events associated mainly with gastrointestinal discomfort. The use of CLA to reduce body weight was investigated by Blankson et al. [205] in a randomized double blind study. In the study 47 overweight subjects completed the study over a 12 week period and it was concluded that the upper limit of 3.4 g CLA/day significantly reduced body fat mass and did not change lean body mass, body mass index, blood parameters and blood lipid profile. In a study of 24 women of which 17 completed the study, CLA consumption of 3 g CLA/day for 64 days did not change fasting insulin, glucose, lactate, and body weight [129]. The authors noted a transient decrease in leptin, however this was not associated with any alteration in appetite.

8. Future Direction

CLA is an exciting new field of study. Other potential beneficial properties of CLA are currently under investigation. There is evidence of protective morphological effects of CLA in mammary tissue. In female rats, CLA inhibits the differentiation of

terminal end buds, the site of initial cancer insult in mammary tissue [61]. There is evidence indicating that CLA may also promote apoptosis in cancer cells [142; 143] and that maybe mediated by a PPAR [144]. Some work has looked at a possible role for CLA in modulating signal transduction, however current evidence shows that CLA does not modulate signaling via protein kinase C (PKC) and phospholipase C (PLC) [150].

The expansion of research into new areas of studies is very exciting and demonstrates that CLA has wide ranging applications and effects on cell biology, gene regulation and the modulation of disease outcome. This area is still very new and most research is at a basic level. As this field of study continues to grow, important future areas of investigation will emerge. In particular, the elucidation of the role of specific CLA isomers in chronic diseases of cancer, atherosclerosis, diabetes and other related ailments will become a primary focus. In addition, once sufficient basic research has been completed, clinical human studies will be undertaken to move the research forward to evaluate the effectiveness of CLA. Once evidence has been established to identify the biological activity of specific isomers, an area of opportunity that will emerge quickly is in the area of methods development to mass produce specific isomers for both research and commercial uses. Commercial uses will most likely include products for human consumption as a supplement, neutraceutical or functional food.

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Chapter 2 - Study Parameters

1. Rationale

Availability of a good source of CLA in terms of quantity and composition is problematic. Sources of CLA, which include ruminant animal fat, are low in CLA content and commercially available mixtures are variable in composition. Improved synthetic and analytical methods are needed to detect, separate, and identify CLA in biological materials. Analytical obstacles have hindered the separation and identification of the many CLA isomers. The effect of individual isomers is unknown as pure isomers are not readily available. Development of these methods will provide a foundation for the investigation of the mechanism of CLA action.

The mechanism of CLA action is unknown but incorporation of CLA into membrane phospholipid suggests a functional role analogous to LA and AA metabolism in the synthesis of biologically important eicosanoids. The localization of the major CLA isomers, Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 into membrane phospholipid classes is not well understood and has relevance for understanding the impact of CLA on essential fatty acid metabolism and eicosanoid synthesis. CLA action may involve substrate competition, inhibition of enzyme function, and potentially the formation of biologically active products. Elevated production of eicosanoids is a characteristic of cancerous cells, which is a useful model for studying the effect of CLA on essential fatty acid metabolism and tumor outcome. The anti-carcinogenic effect of CLA remains a hot topic of study, especially with breast cancer. A useful model to study the effects of CLA on essential fatty acid metabolism and eicosanoid synthesis is the MDA-MB-231 mammary tumor cell, which has been reported in the literature to produce the eicosanoids PGE_2 and LTB_4.

2. Hypotheses

It is hypothesized that CLA reduces levels of essential fatty acids in membrane phospholipid, subsequently reducing prostaglandin and leukotrienes synthesis.

It is specifically hypothesized that:

1. CLA displaces LA and AA, precursors of eicosanoids from membrane phospholipid, thus reducing the synthesis of the eicosanoids, PGE_2 and LTB_4.

2. CLA is elongated and desaturated to 20 carbon products such as Δ5*c*,8*c*,11*c*,13*t*-20:4 and Δ5*c*,8*c*,12*t*,14*c*-20:4 which compete with AA for cyclooxygenase and lipoxygenase thus reducing the synthesis of the eicosanoids, PGE_2 and LTB_4.

3. CLA derived 20 carbon products metabolized via 5-lipoxygenase yield uncharacterized eicosanoids, predicted to be isomers of LTB_4, such as 14-hydroxy LTB_4 and 10-hydroxy LTB_4.

3. Objectives

It was apparent early on that existing analytical and methodological approaches for the study of CLA were inadequate or non-yet developed. Therefore Objective (1) of this thesis is focused on the development of analytical tools and methods useful for the purposes of testing the hypotheses posed. Existing GLC analyses to separate, identify and quantify CLA isomers is technically challenging. The composition of commercially available CLA is of variable quality and not reliable. Pure or enriched isomers of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2 are not commercially available and methods to synthesize or purify these isomers are lacking. Interestingly, there is very little information about CLA in Canadian foods. The establishment of these methodologies and techniques will be useful for the purposes of determining for the first time, the content of CLA in Canadian beef and dairy foods, valuable information to assess intake and make recommendations by health professionals on CLA consumption.

Objective (2) describes the measures of interest to answer the hypotheses posed. The measures will relate changes in membrane phospholipid composition to changes in the synthesis of mediators of cellular function, eicosanoids, which also regulate cell growth.

(1) Develop methods to prepare CLA isomers.

(a) Synthesize CLA from linoleic acid from vegetable oil

(b) Separate and identify CLA isomers by GLC

(c) Fractionate/enrich for the major CLA isomers, Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2

(d) Determine CLA content in Canadian dairy and beef products

(2) Using the methods established in objective (1) to characterize in a cell line the effect of equal or enriched mixtures of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2 on:

(a) LA and AA content in membrane phospholipid fractions of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI)

(b) Growth effect of these CLA mixtures

(c) Identification of elongated/desaturated products derived from CLA, Δ5*c*,8*c*,11*c*,13*t*-20:4 and Δ5*c*,8*c*,12*t*,14*c*-20:4

(d) PGE_2 synthesis

(e) LTB_4 synthesis.

(f) Isolating and identifying possible eicosanoids derived from CLA.

4. Chapter Format

The hypotheses and objectives proposed are organized in the following chapters as follows:

Chapter 3 fulfills Objective 1a and 1b

To develop a method to synthesize a simple mixture of CLA isomers from safflower oil. Commercially available linoleic acid in high purity is typically used in the synthesis of CLA. It was endeavored to extend this method by determining the feasibility of using a vegetable oil rich in linoleic acid as the starting material. To evaluate the separation of CLA isomers and their identification using a new and highly polar GLC type of column.

Chapter 4 fulfills Objective 1c

To devise a method to separate or enrich for the Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 isomers starting from a mixture of CLA isomers containing predominantly Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 in equal amounts.

Chapter 5 fulfills Objective 1d

To quantify the level and types of CLA isomers present in Canadian dairy and beef products using methods and analytical approaches developed in Chapter 3.

Chapter 6 tests Hypothesis 1, 2 and 3 and fulfills Objective 2a - 2f

The effect of CLA on essential fatty acid metabolism was examined using the MDA-MB-231 mammary tumor cell line. CLA mixtures prepared as described in Chapter 4, having equal amounts of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 or enrichment in Δ9*c*,11*t*- or Δ10*t*,12*c*-18:2 were co-supplemented into culture with LA. Phospholipid membrane fatty acids were analyzed. In separate experiments, the effects of these CLA mixtures were used to assess PGE_2 synthesis by gas chromatography mass spectrometry (GC-MS) and growth by radioactive ^{3}H-thymidine incorporation.

Chapter 7 (tests a hypothesis not originally posed)

It is hypothesized that CLA has direct inhibitory effects on 5-lipoxygenase activity in neutrophil differentiated HL-60 cells. The effect of CLA on leukotriene synthesis was examined using the HL-60 cell line, which readily produces leukotrienes derived from 5-lipoxygenase. The original rationale was to use the MDA-MB-231 cell

line to assess the effect of CLA on both prostaglandin and leukotrienes synthesis. PGE_2 was readily detected, however, recovery and detection of LTB_4 was not successful. The HL-60 cell line could only be used to assess direct competitive effects between AA, LA and CLA on 5-lipoxygenase activity.

Chapter 8 provides a general summary, conclusions and discussion of implications, limitations and future research.

Publications from this Thesis

Chapter 1 and Appendix A

(Portions of this chapter and appendix have been published in)

Ma, D.W.L., Ens, J.G., Field, C.J., and Clandinin, M.T. (2001) Conjugated Linoleic Acid: Methods, Biological Effects and Mechanisms. Research Advances in Oil Chemistry (book chapter) Global Research Net Work. (in press).

Chapters 3

(Portions of this chapter have been published in)

Ma, D.W.L., Wierzbicki, A.A, Field, C.J., and Clandinin, M.T. (1999) Preparation of conjugated linoleic acid from safflower oil. JAOCS, 76, 729-730.

Chapter 5

(Portions of this chapter have been published in)

Ma, D.W.L., Wierzbicki, A.A, Field, C.J., and Clandinin, M.T. (1999) Conjugated linoleic acid in Canadian dairy and beef products. J.Agric.Food Chem., 47, 1956-1960.

Chapter 6

(Portions of this chapter have been submitted to)

Ma, D.W.L., Field, C.J., and Clandinin, M.T. (2001) $\Delta 10t,12c$-18:2 inhibits essential fatty acid metabolism in MDA-MB-231 mammary tumor cells. Submitted to Carcinogenesis.

Chapter 7

(Portions of this chapter have been submitted to)

Ma, D.W.L., Field, C.J., and Clandinin, M.T. (2001) CLA does not inhibit leukotriene synthesis in HL-60 cells. Submitted to Lipids.

Collaborative Publications

(Aspects of this thesis have been used in collaborative work and published in)

1. Ens,J.G., Ma, D.W.L., Cole, K.S., Field, C.J., and Clandinin, M.T. (2001) An assessment of c9, t11 linoleic acid intake in a small group of young Canadians. Nutr.Res. (in press).

2. Jones, S., Ma, D.W.L., Robinson, F.E., Field, C.J. and Clandinin, M.T. (2000) Isomers of conjugated linoleic acid (CLA) are incorporated into egg yolk lipids by CLA-fed laying hens. J. Nutr., 130, 2002-2005.

Chapter 3 - Preparation of CLA from Safflower Oil

1. Introduction

CLA refers to a group of geometrical and positional isomers of LA. CLA is reported to have wide ranging biological effects such as inhibiting tumor growth [1; 2], reducing atherosclerotic risk [3] and reducing body fat [4]. Synthetically prepared mixtures containing predominantly the Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2 isomers in equal amounts have been widely used to study these effects. Alkali isomerization of LA is a common method used to synthesize hundred gram quantities of CLA [5]. The composition is often very complex consisting of *cis/trans* (*c/t or t/c*), *cis/cis* (*c/c*) and *trans/trans* (*t/t*) isomers of Δ9,11-, 10,12- and 11,13-18:2 [6]. Early analytical methods using GLC for the separation and identification of the many isomers of CLA is inadequate [7; 8] and the biologically abundant isomer, Δ9*c*,11*t*-18:2 co-eluted with the Δ9*t*,11*c*-18:2 isomer. Therefore, accurate assessment of this isomer in biological materials is not possible.

The objective of this study is to devise methods to synthesize CLA inexpensively, improve the separation of CLA isomers by GLC and to identify the major isomers present.

2. Materials

Free fatty acids of CLA were obtained commercially (Nu-Chek-Prep, Inc., Elysian, MN, Matreya, ON, Canada, and Natural Lipids, Hovdebygda, Norway as a generous gift from Dr. Clement Ip, Roswell Park, Buffalo). Safflower oil was obtained locally from a grocery store (Edmonton, Alberta, Canada). All other chemical reagents were obtained from Sigma/Aldrich (Oakville, Ontario, Canada)

3. Methods

A. Extraction of LA

LA was extracted from safflower oil, rich in LA [9]. Safflower oil (100 g) was saponified using ethanolic potassium hydroxide (KOH) (23 g in 50 mL) for 1 hr under

reflux in a round bottom flask (250 mL) with stirring. The reaction mixture was allowed to cool before transfer to a separatory funnel (1 L). The reaction mixture was acidified with sulfuric acid (4M, 120 mL) to pH<2. Chilled double deionized water (ddH_2O) (250 mL) was then added. Crude LA was extracted with hexane (250 mL). The aqueous phase was re-acidified with sulfuric acid (4M, 50 mL) then ddH_2O (50 mL) was added, followed by re-extraction with hexane (100 mL). Both hexane fractions were combined, dried over anhydrous sodium sulfate and evaporated using a rotary evaporator. Typically, 90% by weight of material was recovered.

B. Purification of LA

Saturated and monounsaturated fatty acids were removed by co-crystallization in urea [9]. Each 100 g of crude LA was co-crystallized in 120 g of urea dissolved in 240 mL of warm methanol (MeOH) and left over-night at 5°C. The mother liquor was separated from the urea adduct material by vacuum filtration. The mother liquor was transferred to a separatory funnel (1 L) and acidified to pH<2 with hydrochloric acid (HCl) (6N, 200 mL) and ddH_2O (200 mL). LA was extracted with hexane (2 x 100 mL), and dried over anhydrous sodium sulfate. Hexane was removed using a rotary evaporator. Typically, 30-50% yield by weight of material was recovered. The fraction recovered was 95% LA. If LA was not used immediately it was typically stored at –70°C under nitrogen. Additional LA was recoverable from the urea adduct fraction [9]. Adduct material was dissolved in warm ddH_2O. HCl (6N) was then added to acidify the mixture to pH<2. Petroleum ether (2 x 150 mL) was used to extract the oil, and then dried over anhydrous sodium sulfate. The organic solvent was removed using a rotary evaporator. This material is high in saturated and monounsaturated fats, therefore would solidify at room temperature. The recovered material was dissolved in acetone, 1 g/mL and crystallized at -20°C. The crude LA was recovered by vacuum filtration from the mother liquor portion, which was subsequently purified by urea crystallization.

C. Isomerization of LA

The method described by Chin et al. [5] with modifications was used to synthesize CLA from LA purified by urea. Conditions were established by assessing the CLA synthesized at 120-140°C, 160-180°C and >180°C. LA was isomerized at 160°C instead of 180°C as stated in the method by Chin et al. [5]. Typically, ethylene glycol (100 g) was heated to 160°C in a 3 neck round bottom flask (500 mL) to reflux on a heating mantle controlled by a variable resistance meter. The reaction was kept inert by a continuous stream of nitrogen. Excess pressure was bubbled through a water trap. At 160°C, the round bottom was taken off the heating mantle and cooled briefly before 13 g of KOH was added. Once the KOH was dissolved, LA (50 g) was added. The reaction was allowed to proceed at 160°C for 1.5 hr and increased to 180°C for an additional 30 min. The reaction mixture was transferred to a separatory funnel (1 L) and MeOH (200 mL) was used to rinse the round bottom, which was then added to the separatory funnel. The solution was acidified to pH<2 with HCl (125 mL, 6N), then ddH_2O (125 mL) was added. CLA was extracted with hexane (1x 100 mL, 2 x 50 mL) and transferred to a separatory funnel (500 mL). The hexane phase was washed with MeOH-ddH_2O (30%, 3 x 100 mL), then with ddH_2O (3 x 100 mL). The hexane phase was dried over anhydrous sodium sulfate. Hexane was removed using a rotary evaporator and CLA was flushed with nitrogen and stored at -15°C.

D. Spectrophotometric Analyses of LA and CLA

Purified LA was analyzed by IR (model 5-DX FTIR; Nicolet, Madison, Wisconsin) and ^{1}H-NMR (300Mhz NMR; Varian, Los Angeles, California) as free fatty acids. CLA was analyzed by IR, ^{1}H-NMR, ^{13}C-NMR (AM-300Mhz; Bruker, Karlsruhe, Germany), and ultraviolet (UV) spectroscopy (model 8452A Diode Array Spectrophotometer; Hewlett-Packard, USA) as free fatty acids. IR analyses used neat free fatty acids. UV analysis was done using free fatty acids dissolved in MeOH. NMR analyses were done in deuterated chloroform.

E. Assessment of Methylation Conditions

Synthetic CLA was used to assess methylation conditions recommended by Werner et al. [10]. A total of 6 determinations were made for each combination of temperature (25°C or 100°C) and duration (30 min or 60 min). Each determination consisted of CLA (1 mg), and C15:0 free fatty acid (50 ug) internal standard in a 9 mL screw cap tube. 14% BF_3-MeOH (1 mL) and hexane (2 mL) was added to each sample. Samples were methylated at room temperature with vigorous shaking or in a 100°C sandbath for either 30 or 60 min. Then ddH_2O (1 mL) was added, vortexed briefly and then centrifuged at 300 x g for 10 min to separate phases. The upper hexane phase was transferred to a GLC vial and evaporated under nitrogen with gentle heating. Samples were then analyzed by GLC for total CLA content and isomeric differences.

F. Gas Liquid Chromatographic Analyses of LA and CLA

The analysis of methyl esters was carried out with a Varian 6000 gas chromatograph running the Varian Star Chromatography Workstation (version 4.0). LA was analyzed on a BP-20 capillary column (25 m x 0.25 mm i.d.; 0.25 μm film thickness) (Supelco Inc., Bellefonte, PA) [11]. Samples were dissolved in hexane. The injector port temperature was 250°C. The detector temperature was 270°C. The carrier gas was helium with 1.5 mL/min flow rate. The column pressure was 30 psi. A 100:1 split mode was used. Samples were eluted off the column using a temperature program starting at 90°C and held for 2 min, increased to 178°C (25°C/min) and held for 8 min, then increased to 220°C (25°C/min) and held for 0.18 min. A FID detector was used. Materials containing CLA were analyzed using a SP-2560 fused silica capillary column (100 m x 0.25 mm i.d., 0.2 μm film thickness; Supelco Inc, Bellefonte, PA). Samples were dissolved in hexane. Column conditions were set as follows, the injector and detector ports were set to 250°C and 270°C, respectively. The carrier gas was helium with 2 mL/min flow rate. The column pressure was 50 psi. A 100:1 split mode was used. Samples were eluted off the column using a temperature program starting at 130°C, increased to 150°C (20°C/min)

and held for 5 min, increased to 165°C (20°C/min) and held for 60 min, then increased to 225°C (2°C/min) and held for 13.25 min. A FID detector was used.

G. Argentation Thin Layer Chromatography

Thin layer chromatography (TLC) H-plates (20 x 20 cm) were impregnated with silver (2.5% wt/vol, 1.25 g $AgNO_3$ / 50 mL ddH_2O) by placing the plate vertically into a TLC tank containing an aqueous solution of silver nitrate for 1 hr. A plate impregnated using a solution of silver nitrate in this manner deposits approximately twice as much silver by weight. Therefore, a 2.5% solution of silver nitrate will deposit 5% silver by weight of silica. The distribution of silver was greater near the bottom of the plate and at the leading edge of the silver front and uniform in the middle portion. After impregnating with silver, plates were activated at 110°C in an oven to remove water. To effect separation on TLC, CLA fatty acids were converted to methyl esters. Methyl esters of CLA were prepared by dissolving in hexane (2 mL) free fatty acids of CLA (1 mg) and methylating with 14% BF_3-MeOH (1 mL) for 30 min at room temperature with shaking. The hexane phase was recovered and reconstituted to a known volume. CLA methyl esters were spotted 2 cm from the bottom edge of the plate (25 μg/cm). Plates were developed until the solvent front reached the top of the plate. Plates were developed using solvent mixtures containing chloroform. An upper *t/t* band and a lower *c/t* ; *t/c* were separated by double development with hexane : chloroform (90 : 10) followed by hexane : chloroform (10 : 90). Similar results were be obtained with a single development with hexane : chloroform (50 : 50). Bands were visualized by charring or by UV detection (366 nm) after spraying with 2,7-dichlorofluorescein in MeOH (0.025% w/v). UV detected bands were scraped into 9 mL screw cap tubes containing ddH_2O (1 mL). Methyl esters were extracted with hexane (2 x 2 mL) and subsequently analyzed by GLC. The methylated upper band co-chromatograms with a known standard of Δ9*t*,11*t*-18:2 by GLC. The lower broad band contained a mixture of *c/t* and *t/c* isomers upon analysis by GLC

H. Enrichment of a Single CLA Isomer

CLA prepared as previously described was enriched for a single isomer of CLA. The amount of urea used was based on recommendations by Gunstone et al. [9] as the % unwanted material x weight material x urea factor. Starting with 4 g of CLA, 10 g of urea was needed (0.5% x 4 g x 5). A solution of urea-MeOH (10 g/50 mL) was prepared with heating in an erlenmeyer flask (250 mL). CLA (4 g) was added and allowed to crystallize for 2 days at 4°C. The urea adduct was filtered by vacuum, and washed with cold saturated urea-MeOH (50 mL). The mother liquor was transferred into a separatory funnel (250 mL). The collection flask was washed with ddH_2O (50 mL) and added to the separatory funnel. Fatty acids were acidified to pH<2 with HCl (6N, 50 mL). CLA was extracted with hexane (50 mL). The aqueous phase was re-extracted with successive hexane washings (25 and 10mL). The organic phase was washed with MeOH-ddH_2O (30%, 3 x 25 mL), then with ddH_2O (3 x 25 mL) and dried over anhydrous sodium sulfate. The organic solvent was removed using a rotary evaporator. The recovered material was re-crystallized a second time using the same procedure.

I. Hydrazine Reduction of Octadecadienoates

The method for the partial hydrazine reduction of fatty acids was modified from the method described by Ratnayake et al. [12]. Octadecadienoic fatty acids of Δ9*c*,12*c*-, Δ9*t*,11*t*- and Δ9*t*,12*t*-18:2 (1-10 mg) were transferred into 9 mL screw cap tubes. A 100 fold volume of hydrazine-hydrate in MeOH (10% v/v, 100 µl hydrazine-hydrate and 900 µl of MeOH) was then added. The tube was capped and then shaken at 50°C for 1.5 hr. The incubation time can be varied between 0.5-2.0 hr depending on the type of material being analyzed to effect partial or total reduction of all double bonds. HCl (6N, 1.5 mL) was added to stop the reaction, then ddH_2O (1 mL) and hexane (2 mL) were added and the mixture was centrifuged at 300 x g for 10 min to permit phase separation. The upper hexane phase was transferred to a clean 9 mL screw cap tube and dried under nitrogen with mild heating. The aqueous phase was re-extracted with hexane (2 mL) and combined, but not dried down. The free fatty acids were methylated by addition of 14% BF_3-MeOH (1 mL) and heated in a sandbath for 1 hr at 110°C. The mixture was then

cooled (5-10 min) and ddH_2O (1 mL) was added and centrifuged at 300 x g for 10 min to separate phases. The upper hexane phase was transferred to a GLC vial and subsequently analyzed by GLC.

J. Identification of Δ9c,11t- and Δ10t,12c-18:2 CLA

Monoene standards of Δ9*c*-, 9*t*-, and Δ11*c*-18:1 purchased commercially were combined with hydrazine reduced octadecadienoates to complete a library of positional and geometrical isomers of Δ9, Δ11, and Δ12 with cis or trans geometry. These monoenes were separated by GLC using a SP-2560 fused silica capillary column (100 m x 0.25 mm i.d x 0.2 μm film thickness; Supelco Inc, Bellefonte, PA). Column conditions were set as follows, samples were dissolved in hexane, the injector port was set to 250°C, detector temperature was 270°C, carrier gas was He, column pressure was 50 psi having a flow rate of 2 mL/min and a 50:1 split mode. Samples were eluted off the column using a temperature program starting at 90°C, increased to 150°C (15°C/min) and held for 8 min, increased to 160°C (20°C/min) and held for 76 min, then increased to 225°C (5°C/min) holding for 23 min. A FID detector was used for sample detection.

CLA enriched for a single isomer was subjected to hydrazine reduction as described previously. Retention times and abundance of CLA monoenes were compared to the established monoene library.

4. Statistical Analysis.

Intraisomerization resulting from methylation conditions of time and temperature were analyzed by two-way analysis of variance (ANOVA) analysis. Fatty acid profiles derived from GLC analyses of safflower oil, enriched LA, and CLA are expressed as the mean ± SEM from multiple preparations.

5. Results

A. Purification of LA

Safflower oil was chosen as a source of LA because of its high content, approximately 75% (wt/wt). TLC was used to monitor the extent of the saponification, which showed complete liberation of free fatty acids from its esterified triacylglycerol form. Compositional information of crude, purified and isomerized LA is summarized (Table 3-1). Safflower oil contained 76.4% LA with the remainder consisting of 10.2% saturated, and 13.2% mono-unsaturated / polyunsaturated fatty acids by GLC. After urea purification the level of LA was enriched to 95.1 and 96.9%, in the mother liquor and urea adduct fraction, respectively. Typically, the fraction most easily enriched was the mother liquor, which required only a single enrichment step, with yields >50%, by weight. The adduct fraction required both urea and low temperature enrichment steps, and produced low yields. LA was confirmed as the major fatty acid after enrichment of safflower oil by urea purification as indicated by IR (Figure 3-1A) [13; 14], ^{1}H-NMR (Figure 3-2A) [15; 16] and GLC (Figure 3-3B) by comparison to a known standard.

B. Synthesis of CLA

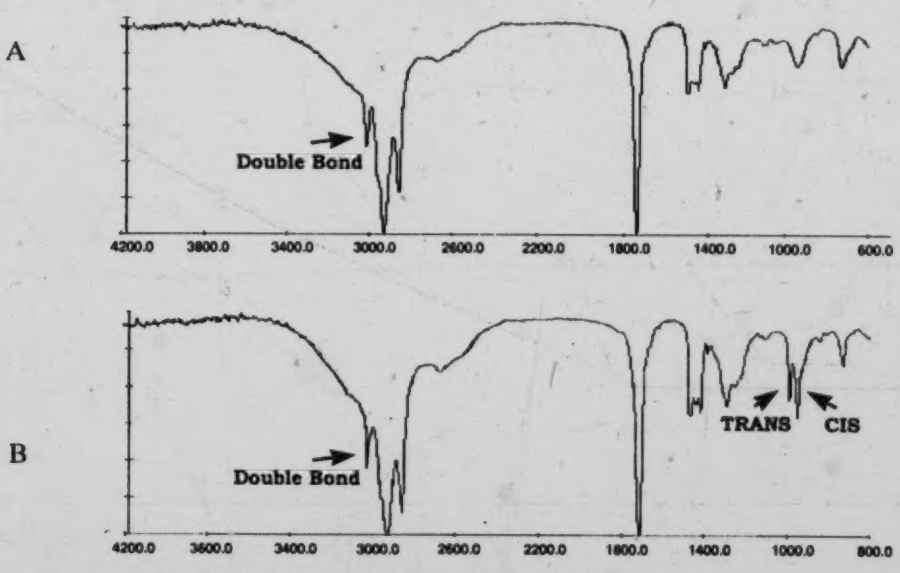
Alkali isomerization of LA over 2 hrs at 120-140°C, 160-180°C and >180°C was assessed for conversion of LA to CLA. Isomerization carried out at 120-140°C was incomplete, with approximately 60% of the LA remaining. Both the 160-180°C and >180°C resulted in near conversion of all LA to CLA, with 1-2% LA remaining. Different isomer profiles were obtained at each temperature (Figure 3-4). As the reaction temperature increased from 120-140°C to 160-180°C to >180°C, levels of the two major isomers, peak 1 and 4 progressively decreased from 98% to 91% to 82% (% of total CLA), respectively. Concomitantly, new isomers were formed and the level of these minor isomers progressively increased. All CLA was prepared at 160-180°C, which was the minimal temperature yielding complete conversion of LA to CLA within 2 hrs with good reproducibility.

Table 3-1 Fatty Acid Composition of Safflower Oil, Purified LA and CLA

	Saturates	Mono/Poly[5]	LA	CLA	N	%yield[6]
Safflower Oil	10.2 ± 0.1	13.2 ± 1.5	76.4 ± 1.5	0.2 ± 0.01	5	93
Purified LA[1]	0.9 ± 0.8	4.0 ± 2.4	95.1 ± 1.6	0	2	36-58
Purified LA[2]	0.8 ± 0.7	2.3 ± 0.8	96.9 ± 1.5	0	1[7]	21
Isomerized LA[3]	2.0 ± 0.6	4.5 ± 0.4	2.3 ± 0.8	91.2 ± 0.8	6	30-52
Isomerized LA[4]	0.9 ± 0.1	4.6 ± 0.3	0.9 ± 0.02	93.6 ± 0.2	2	17.8

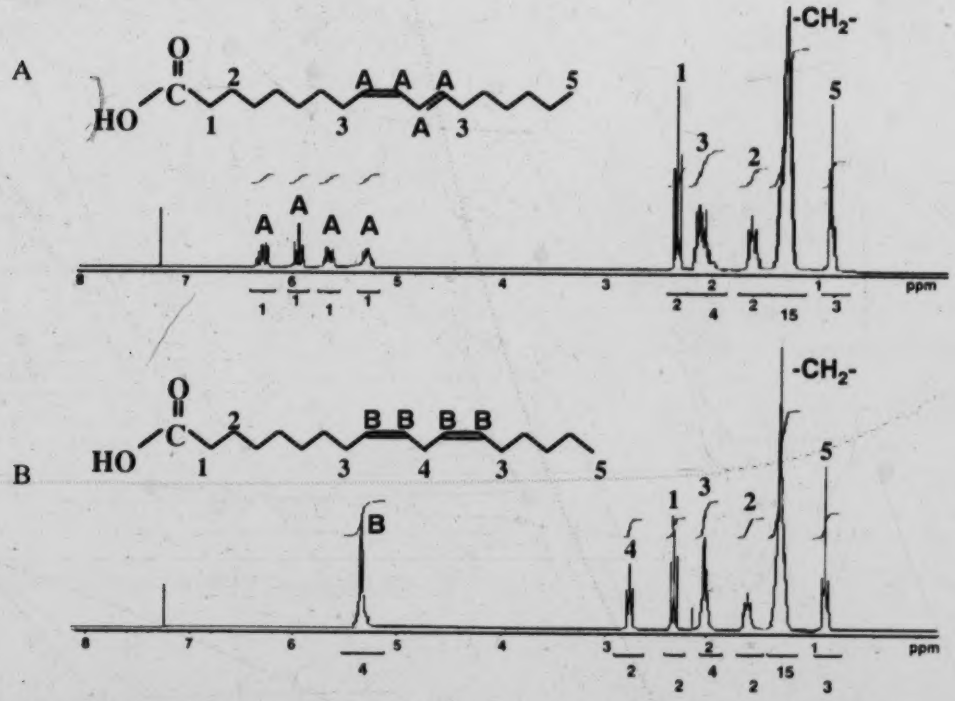
[1,2] LA recovered from the urea mother liquor and adduct fraction, respectively.
[3,4] LA from the urea mother liquor and adduct fractions isomerized to produce CLA, respectively
[5] Monounsaturated/Polyunsaturated fatty acids do not include LA
[6] Yields are with respect to the original starting weight of safflower oil
[7] The error refers to replicate measurement of the single batch by GLC.
* Values represent the percent area count ± SEM from gas chromatographic analysis from either a BP20 or SP2560 GC column

Figure 3-1 Infrared Spectra of LA and CLA.



Strong peaks at 3020 and 3040cm^{-1} (C-H stretch) indicates double bond structure for LA (panel A) and CLA (panel B) respectively. Medium strength peaks indicate cis and trans geometry of the conjugated double bond structure of CLA at 960 and 1000cm^{-1} (out of plane bend) respectively.

Figure 3-2 ^{1}H-NMR Profile of LA and CLA

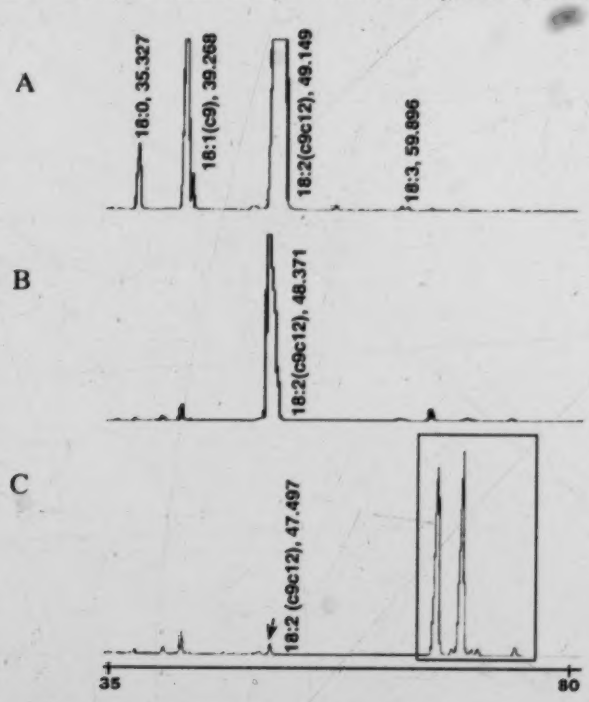


Numbers above peaks correspond to the specific carbon indicated in chemical formula. Spectra was obtained from a 300 MHz instrument. Values reported provide the chemical shift, number of protons, and signal split (multiplet;m, triplet;t, quadruplet;q) associated with each distinct group of peaks. CLA (panel A) consisted of peaks at 0.84-0.89(3,m), 0.92-1.64(15,m), 1.59-1.64(2,m), 2.04-2.15(4,m), 2.33(2,t), 5.22-5.33(1,m), 5.58-6.68(1,m), 5.92(1,t), 6.21-6.2(1,m). LA (panel B) consisted of peaks at 0.87(3t), 1.26-1.36(15,m), 1.59-1.64(2m), 2.03(4,q), 2.33(2,t), 2.75(2,t), 5.31-5.35(4,m).

Compositional information of CLA synthesized is summarized (Table 3-1). Alkali isomerization of LA recovered from the mother liquor and adduct fraction produced CLA enriched to 91.2 and 93.6% (wt/wt), respectively. Total yield of CLA obtained from the enriched LA of the urea mother liquor fraction ranged between 30-52% (wt/wt). Total yield of CLA obtained from LA recovered from the urea adduct fraction was, 17.8% (wt/wt). The product, CLA produced by alkali isomerization of purified LA was identified by IR (Figure 3-1B) [13; 14; 17], ^{1}H -NMR (Figure 3-2B) [7; 16; 18], ^{13}C-NMR (Figure 3-5) [19], UV (Figure 3-6) [8; 14] and GLC (Figure 3-3C). ^{1}H-NMR analysis detected 4 peaks with chemical shifts in the range of 5-6 ppm for double bonds.

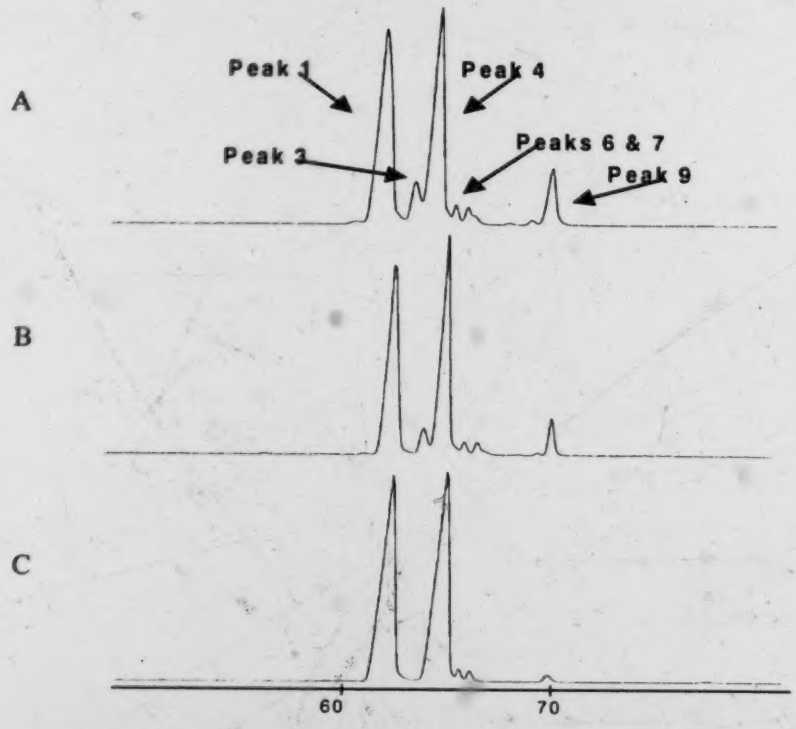
A similar result can be drawn from ^{13}C-NMR analysis, which detected 4 strong peaks with chemical shifts in the range of 125-134 ppm for double bonds. IR analysis indicated the presence of conjugated dienes having both cis (960 cm^{-1}) and trans (1000 cm^{-1}) geometry. A strong UV peak at 234 nm also indicated the presence of a conjugated diene structure. UV 2nd derivative [20] analysis reveals 4 peak minimas, indicating the presence of multiple isomers.

Figure 3-3 GLC Profile of Safflower, Enriched LA, and CLA Mixture



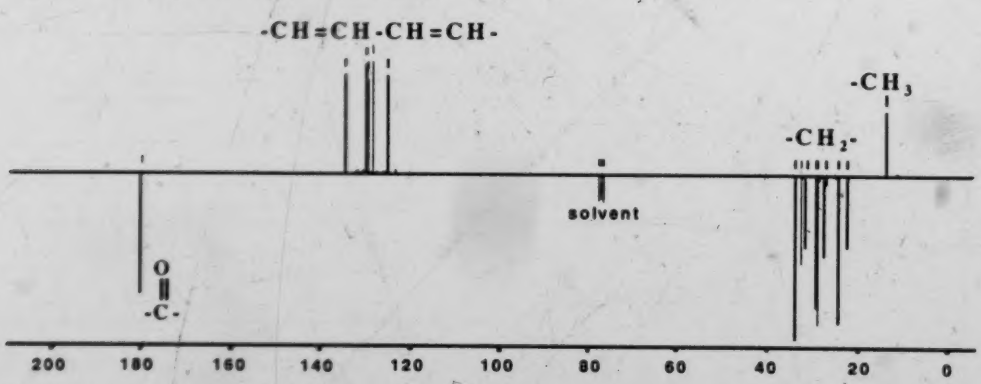
Gas liquid chromatography using a SP-2560 column was used to monitor purity and the identification of CLA isomers. Gas chromatogram of the starting material safflower oil contains predominately LA, Δ9*c*,12*c*-18:2 (panel A). Safflower oil was saponified and subsequently purified using urea that produced an enriched LA product (panel B). The enriched LA was treated with alkali to produce CLA, enclosed in the boxed area (panel C).

Figure 3-4 Compositional Changes of CLA at Various Temperatures



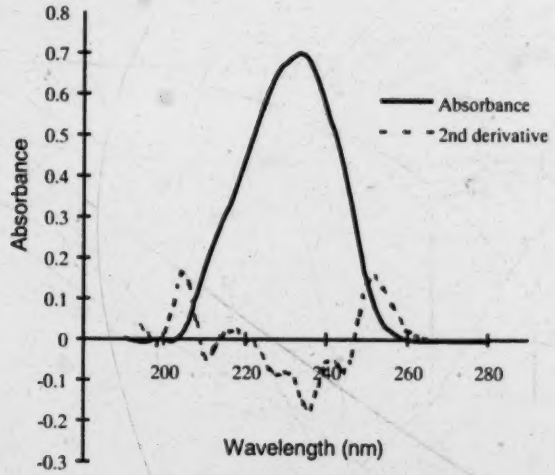
Comparison of the isomer profile at 180°C (panel A), 160-180°C (panel B), and 120-140°C (panel C). As the isomerization temperature increases, the number of minor CLA isomers (peaks 3, 6, 7, and 9) increases due to intraisomerization of the two major isomers (peaks 1 and 4).

Figure 3-5 ^{13}C-NMR Profile of CLA



^{13}C-NMR profile of CLA. Functional and structural components of CLA are indicated by the chemical formulae associated with each peak. Spectra were obtained from a 300 MHz instrument. Chemical shifts are given in ppm units and peaks were observed at 14, 22-34, 125-134, 180. Numerous overlapping peaks were observed, indicating the presence of multiple CLA isomers.

Figure 3-6 UV Profile of CLA



Ultraviolet and 2nd derivative spectra of CLA measured in MeOH. Maximum absorbance and 2nd derivative minimum was observed at 234nm have a molar absorptivity of $\varepsilon=2.4x10^4$. The 2nd derivative spectra contains 4 minimums, indicating that a mixture of conjugated fatty acids is present.

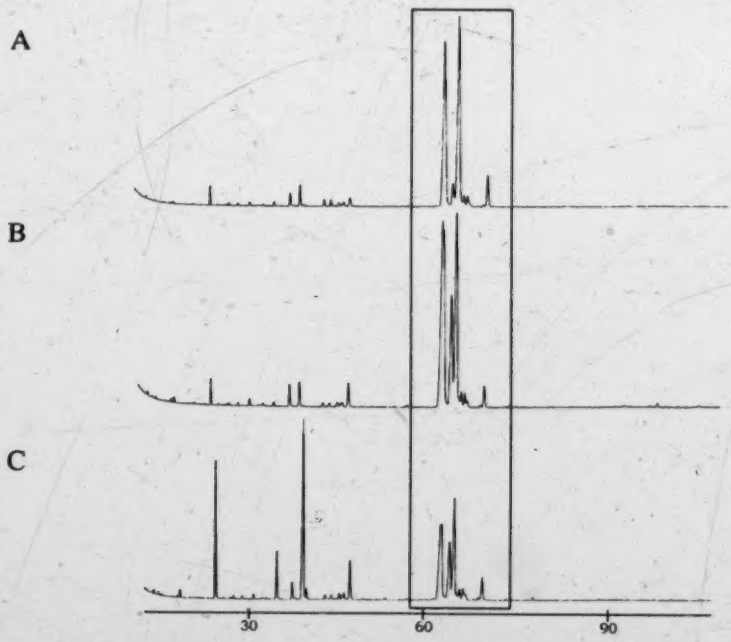
C. Methylation Conditions

Using CLA prepared from safflower oil, methylation conditions were assessed for completeness and intraisomerization. Sustained methylation for 1 hr at 110°C compared to 30 min at room temperature using 14% BF_3-MeOH showed that there was incomplete methylation of monounsaturated and saturated fatty acid at room temperature. There were small differences observed in the distribution of CLA isomers at the higher temperature. To investigate further, a mixture of CLA was methylated at either room temperature (on the lab bench) or 100°C (sandbath) for either 30 min or 60 min durations. CLA methylated at 100°C versus room temperature resulted in non significant loss of the Δ9*c*,11*t*-18:2 isomer (p=0.09). There was no interaction, but both time (p=0.05) and temperature (p=0.02) significantly reduced levels of Δ10*t*,12*c*-18:2. With the loss of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 isomers, there was a concomitant increase in the level of the Δ9*t*,11*t*-18:2 isomer (p=0.008) with temperature. Consequently, all analyses were done at room temperature to avoid intraisomerization.

D. Separation and Identification of the Major CLA Isomers

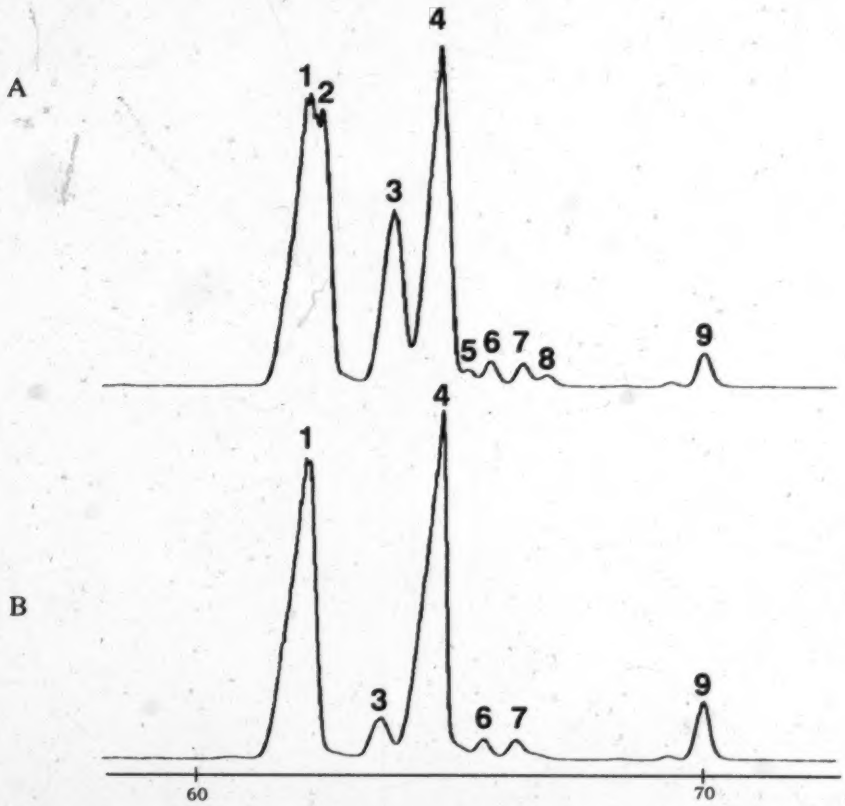
The GLC spectrums of two commercially available CLA standards were similar to that obtained for the safflower derived CLA (Figure 3-7), which elute between 62 and 70 min. However, the commercial CLA mixtures contained three additional peaks (Figure 3-8) for a total of 9 peaks. These new peaks are presumably isomers of CLA since they elute within the narrow range corresponding to the already identified CLA isomers. In the mixture of isomers synthesized from safflower oil, there are 2 major isomers, peaks 1 and 4, and 4 minor isomers, peaks 3,6,7 and 9. As a percentage of the total mixture, peaks 1, 3, 4, 6, 7 and 9 correspond to 45.0±0.7, 3.1±0.7, 46.1±1.0, 0.9±0.04, 1.4±0.1, and 3.5±0.7%, respectively (n=4). In the commercial mixtures, there are 9 isomers present and peaks 1, 2, 3, 4, 5-8 and 9 comprise 31.5±4.5, 11.3±1.6, 17.3±0.8, 32.2±0.7, 3.8±1.1 and 3.8±1.7% respectively, of the total mixture.

Figure 3-7 GLC Profiles of Safflower Derived CLA and Commercial Mixtures



GC chromatograms of safflower oil derived CLA (panel A) and two commercially available mixtures of CLA (panel B and C). CLA isomers are enclosed within the boxed area.

Figure 3-8 GLC Expansion of the CLA Peaks



Gas chromatographic expansions of CLA isomers from a SP-2560 fused silica GLC column. Commercially available CLA mixtures contains 9 peaks (panel A). As a percentage of the total mixture, peaks 1,2,3,4, 5-8 and 9 correspond to 31.5±4.5, 11.3±1.6, 17.3±0.8, 32.2±0.7, 3.8±1.1 and 3.8±1.7%, respectively (n=2). CLA produced from LA derived from safflower oil was separated into 6 peaks (panel B). As a percentage of the total mixture, peaks 1,3,4,6,7 and 9 correspond to 45.0±0.7, 3.1±0.7, 46.1±1.0, 0.9±0.04, 1.4±0.1, and 3.5±0.7%, respectively (n=4). Peaks were integrated from baseline to baseline.

Methods were employed to identify the major isomers contained within the mixture synthesized from safflower oil. Argentation separation of the CLA isomers resolved an all trans band containing a single CLA isomer, which co-eluted with a commercially obtained standard by GLC. The two remaining major peaks of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 remained unresolved as overlapping bands on argentation TLC plates.

Table 3-2 Monoene Library of Positional and Geometrical Isomers of Δ18:1.

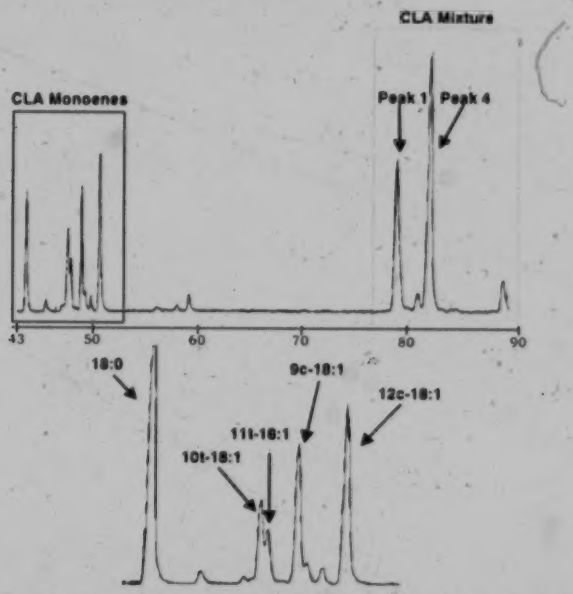
18:1 Monoene	Relative Retention Times with Respect to 18:0 Standards	Values Observed for CLA Derived Monoenes[1]
trans 9	1.0843	1.0854 - 1.0895
trans 10	1.0914*	1.0918 – 1.0930
trans 11	1.0992	1.0997 – 1.1034
trans 12	1.1128	nd
cis 9	1.1207	1.1191 – 1.1236
cis 10	1.1337*	1.1321 – 1.1357
cis 11	1.1418	1.1438 – 1.1461
cis 12	1.1610	1.1599 – 1.1628

[1] Range of values determined from replicate measurements from 3 determinations
[2] Mean retention time of at least triplicate measurements of single standards or of monoenes derived from hydrazine treated octadecadienoates
* Predicted relative retention time
nd = not detected

The major CLA isomers were identified from monoenes produced by partial hydrazine reduction of a mixture enriched for a single isomer (Figure 3-9). Urea crystallization was used to enrich for a single isomer. The resultant mixture of CLA contained 35%, 60% and 5% of peaks 1, 4 and all others. Peak 4 was enriched 1.7 times greater than peak 1. A library of Δ18:1 positional and geometrical isomers was established for Δ9, Δ11, and Δ12 for both cis and trans geometry (Figure 3-10). The relative retention times of these standards were used to derive the log transformed regression equation for each of the cis and trans series, which are y=0.005x+0.0045 and y=0.0036x+0.002, respectively (Figure 3-11). The unknown relative retention times for Δ10 geometrical isomers were predicted. Relative retention times for standards and CLA derived monoenes are summarized (Table 3-2). This library was used to identify the monoenes derived from CLA. Hydrazine reduction of the enriched CLA mixture produced small amounts of Δ8*t*-, Δ9*t*-, Δ12*t*-, Δ10*c*- *and* Δ11*c*-18:1 (Figure 3-12). The monoenes of Δ10*t*-, Δ11*t*-, Δ9*c*-, and Δ12*c*-18:1 were produced in greatest abundance (Figure 3-9). Hydrazine reduction of trans double bonds proceeds with greater ease

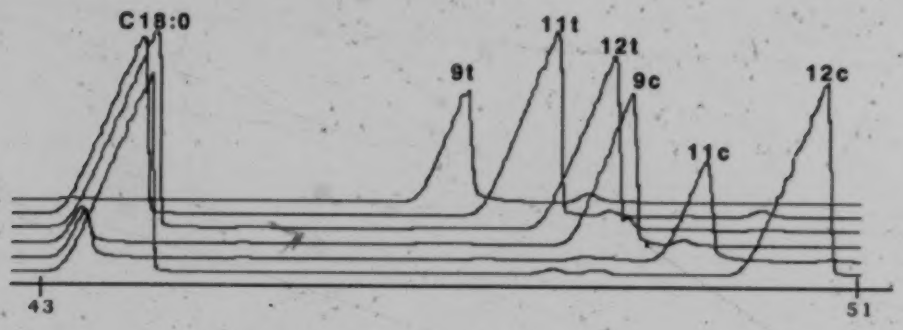
compared to cis double bonds [21]. Hence, trans monoenes are more readily converted to the fully saturated stearate, 18:0. There was 1.5 fold greater amounts of Δ10*t*-18:1 compared to Δ11*t*-18:1, and 1.4 fold greater amounts of Δ12*c*-18:1 compared to Δ9*c*-18:1 (Figure 3-9). The two monoenes in greatest abundance, Δ10*t*- and Δ12*c*-18:1 must derive from peak 4, the enriched peak, and must also be paired in a conjugated manner consistent with the conjugated structure of CLA. Therefore, peaks 1 and 4 are identified as the Δ9*t*,11*c*-18:2 and Δ10*t*,12*c*-18:2 isomers.

Figure 3-9 GLC Profile of Hydrazine Treated CLA



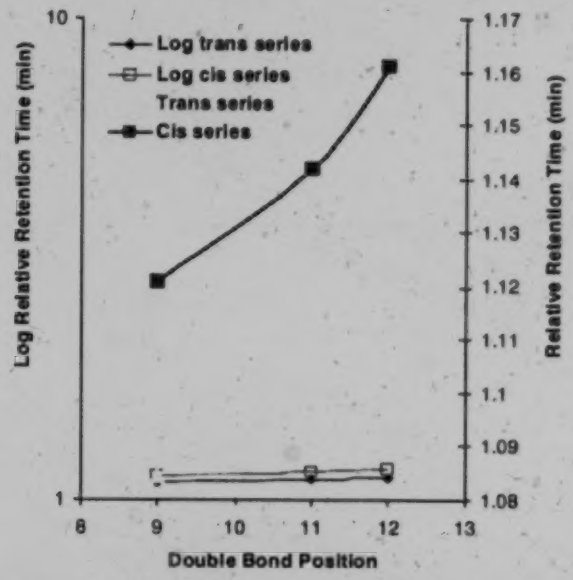
Treatment of a mixture of CLA isomers produces monoenes for each double bond with retention of geometry (upper panel). The upper right box encloses those peaks corresponding to the enriched mixture of CLA. The upper left box encloses those peaks corresponding to the monoene and saturated C18:0 products derived from hydrazine treatment. Complete hydrazine reaction produces the fully saturated C18:0. There was 1.5 fold greater amounts of Δ10*t*-18:1 compared to Δ11*t*-18:1, and 1.4 fold greater amounts of Δ12*c*-18:1 compared to Δ9*c*-18:1 Since the monoenes are derived solely from conjugated double bonds, the monoenes must be paired similarly. Therefore, the only logical pairings are the Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2, which correspond to peaks 1 and 4 respectively.

Figure 3-10 GLC Profile of Monoene Standards



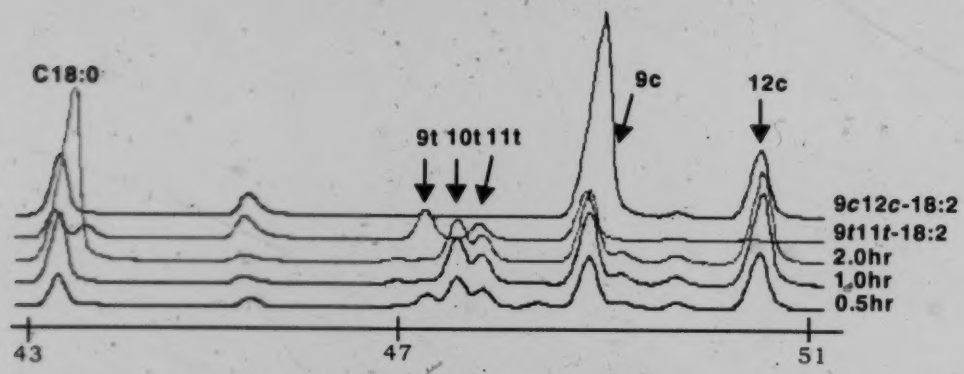
C18:0 elutes at 43 min and is used as a reference peak. The first monoene trans Δ9*t*-18:1 elutes at 47 min, and the Δ12*c*-18:1 elutes last at 50 min.

Figure 3-11 Normal and Log Plots of Geometrical and Positional Isomers of Δ18:1



Positional and geometrical monoenes derived from octadecanoic acids or hydrazine treated octadecadienoic acids were analyzed on a SP-2560 GLC capillary fused column. A total of 6 monoenes were used to construct cis and trans series consisting of Δ9, Δ11 and Δ12 positional isomers. The relative retention times with respect to C18:0 were log transformed and used to derive the regression equation for the cis and trans series of monoenes. The regression equations for the cis and trans series are y=0.005x+0.0045 and y=0.0036x+0.002, respectively. The regression equation was used to predict the retention time for the cis and trans Δ10 isomers.

Figure 3-12 Hydrazine Treated CLA and Octadecadienoates



Monoenes from Δ9c12*c*-, and Δ9*t*,11*t*-18:2 aid in the identification of monoenes produced from hydrazine treatment of CLA. Treatment of CLA at various times, show that Δ10*t*, Δ11*t*, Δ9*c* and Δ12*c* are present in greatest abundance. At 0.5 hr, minor amounts of Δ8*t*, Δ9*t*, Δ12*t*, Δ10*c*, and Δ11*c* are detected and are trace amounts remain after 2.0 hr.

6. Discussion

CLA research has expanded rapidly since its initial isolation and identification in fried ground beef [7; 22]. CLA is commonly synthesized from LA [1; 5]. A method was developed to prepare CLA from a raw material rich in LA such as safflower oil. This has relevance for the synthesis of readily available material for research purposes using inexpensive reagents and readily available equipment. A high purity CLA mixture was readily obtained from safflower oil with good yield. LA was purified to 95% from safflower oil containing 75% LA (Table 3-1). This purified LA was alkali isomerized to produce a mixture of CLA isomers. Total yield from the safflower oil source varied from 30-52.3% or 40-70% of the available LA. The purity of the CLA mixture was 91.2-93.6%. Loss of material occurred primarily in the urea crystallization step. Further recovery of material was possible from the urea adduct fraction. The purified LA and CLA product was confirmed by spectral analysis and gas chromatography. Literature values confirmed the results obtained by IR, ^{1}H-NMR, ^{13}C-NMR, and UV. Gas chromatography of commercial CLA is similar in isomer profile to the safflower derived CLA, but also contained several additional isomers.

Overall, the method described for the enrichment and isomerization of LA to CLA is simple and provides an excellent yield of high purity CLA (Figures 3-3A, 3-3B, 3-3C). Purity is similar to that commercially obtained or better as compared by GLC (Figure 3-7). A similar method described by Wong et al. [23] also shows that safflower oil is a suitable starting material for synthesis of CLA. However, this method has been adapted for the synthesis of CLA directly from safflower oil without enrichment for LA. The procedures described herein have application for the production of CLA for use in culture and animal studies as a source of high purity CLA.

This study shows that the SP-2560 column is capable of improved resolution of CLA isomers not previously separated by regular columns [8]. These additional isomers identified from commercial mixtures of CLA may correspond to newly detected isomers of Δ8,10- or Δ11,13-18:2 reported by Christie et al. [24]. It was initially reported by Ha et al. [8] that a synthetic mixture of CLA derived from LA consists of 9 isomers corresponding to 7 peaks by GLC. The SP-2560 column was able to separate 9 peaks from two commercially available CLA mixtures (Figure 3-8).

The mixture of CLA isomers, which was synthesized at 160-180°C contained 6 peaks (Figure 3-8). Three major isomers were identified from this mixture. Peaks 1, 4 and 9 were identified as the Δ9*c*,11*t*-, Δ10*t*,12*c*- and Δ9*t*,11*t*-18:2 CLA isomers and accounted for 45.0±0.7, 46.1±1.0 and 3.5±0.7% of the total CLA mixture, respectively. Typically, commercial mixtures containing peaks 1, 2 and 4 are reported as 2 peaks identified as Δ9*c*,11*t*-/Δ9*t*,11*c*-18:2, and Δ10*t*,11*c*-18:2, which account for ~90% of CLA content. Two commercial mixtures were analyzed and the Δ9*c*,11*t*-, and Δ10*t*,12*c*-18:2 isomers account for 31.5±4.5 and 32.2±0.7% of the total CLA mixture. Comparatively, these two isomers account for 91.2% or 63.8% of total CLA, in the mixture produced in this study and commercial mixtures, respectively. Levels of the biologically abundant isomer, Δ9*c*,11*t*-18:2, are also markedly different, 45.0% versus 31.5% in commercial mixtures. An isomer identified as peak 2 (Figure 3-8) from the commercial mixtures overlaps that of the Δ9*c*,11*t*-18:2 isomer. This maybe the Δ9*t*,11*c*-18:2 isomer identified by Ha et al. [8], which was not previously separable, and accounts for 11.3±1.6% of the mixture. Therefore, depending on the source or method by which CLA is obtained or synthesized,

varying amounts of the Δ9*c*,11*t*-18:2 isomer may be present and may not be present in largest proportion. Therefore, commercial mixtures may contain less of the Δ9*c*,11*t*-18:2 than is actually reported. This is an important consideration and a source of experimental variation amongst similar experiments, which either utilize commercial or self synthesized mixtures of CLA.

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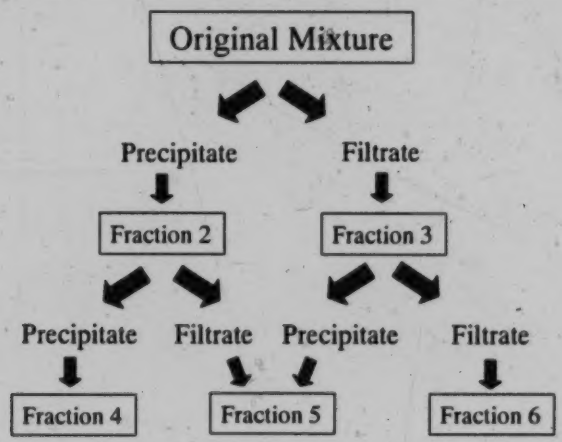
Chapter 4 – Counter Current Separation of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2

1. Introduction

Alkali isomerization of LA is a common method used to synthesize hundred gram quantities of CLA [1]. This was readily accomplished in Chapter 3 and safflower oil was shown to be a useful starting material. The production of single isomers is not as easily accomplished. The use of CLA mixtures in studies prevents the identification of the biologically active isomer. There are limited methodologies to synthesize specific CLA isomers, particularly the Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2 isomers. Several methods describe the synthesis or preparation of these isomers by low temperature crystallization [2], selective lipase esterification [3], directly from methyl ricinoleate [4-6], and by bacterial synthesis [7]. There are some limitations to these methods, which include low yield, and to some degree difficulties in implementation.

Figure 4-1 Conventional Low Temperature Fractional Crystallization Scheme



Separation of fatty acids can be readily accomplished by fractional crystallization procedures (Figure 4-1) [8]. Typically, fatty acid mixtures are crystallized in various types of organic solvents at varying low temperatures. The specific combination of organic solvent and temperature results in selective crystallization of specific fatty acids. This conventional type of crystallization occurs in a single phase. A variation of this

conventional system is the counter-current system, which entails partitioning of two similar compounds with slightly different affinities between two different phases. For example, a mixture of two compounds "A" and "B" have 60% affinity for phase "X" and "Y", respectively. These phases are also immiscible in each other. Compounds A and B will partition such that 60% of A will partition into X and 40% into B and similarly 40% of B partitions into X and 60% partitions into Y. The two phases are then separated and the process is repeated until the desired purity is obtained.

In chapter 3, a method was described to prepare a mixture of CLA isomers enriched in Δ10*t*,12*c*-18:2 by urea crystallization. This suggests that a systemic approach can be devised to prepare enriched mixtures of both Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2. Described herein is a counter-current approach to prepare enriched mixtures of either the Δ9*c*,11*t*- or Δ10*t*,12*c*-18:2 isomer by urea crystallization.

2. Materials and Methods

A. Preparation of CLA

A mixture of isomers containing predominantly Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 in equal proportion was prepared from safflower oil as described in chapter 3.

B. Urea Crystallization of CLA

CLA (50 g) was crystallized with an equal weight of urea dissolved in warmed methanol (MeOH) (2 g/mL). The urea must be fully dissolved to ensure effective crystallization. CLA rapidly crystallizes and is allowed to cool at room temperature or 5°C briefly before chilling overnight at –25°C. Next day, the urea crystal (UC) fraction was separated from the mother liquor (ML) fraction by vacuum filtration. The urea crystal fraction was washed with chilled saturated urea in MeOH and pressed with a glass stopper to dryness.

C. Extraction of CLA from ML Fraction

The ML fraction was transferred to a separatory funnel, ddH_2O (50 mL) was added and the mixture was acidified with HCl (6N, 50 mL) to pH<2. CLA was extracted with hexane (2 x 50 mL) and transferred to a clean separatory funnel. The hexane fraction was washed with ddH_2O (25 mL) acidified with HCl (6N, 25 mL). The hexane fraction was further washed with MeOH in ddH_2O (30% v/v, 3 x 50 mL) and ddH_2O (3 x 50 mL). The hexane fraction was dried over anhydrous sodium sulfate and removed by rotary evaporator.

D. Extraction of CLA from UC Fraction

The UC fraction was dissolved in ddH_2O (50 mL) and heated to dissolve the urea. CLA should appear as a separate distinct upper phase upon complete dissolution of urea. The heated mixture was briefly cooled, then transferred to a separatory funnel. HCl (6N, 50 mL) was added to acidify to pH<2. CLA was extracted with hexane (2 x 50 mL) and transferred to a clean separatory funnel. The hexane fraction was washed with ddH_2O (25 mL) acidified with HCl (6N, 25 mL). The hexane fraction was further washed with MeOH in ddH_2O (30% v/v, 3 x 25 mL) and ddH_2O (3 x 25 mL). The hexane fraction was dried over anhydrous sodium sulfate and removed by rotary evaporator.

E. Enrichment of Δ9c,11t- and Δ10t,12c-18:2

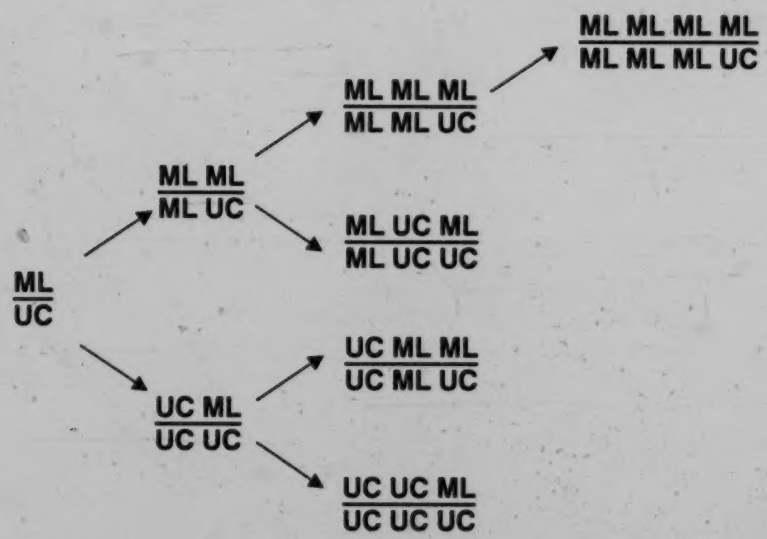
The initial ML fraction, enriched in Δ10*t*,12*c*-18:2 was subsequently recrystallized in equal weight of urea and worked-up similarly as previously described. Each successive ML fraction was again recrystallized. The initial UC fraction, enriched in Δ9*c*,11*t*-18:2 content was subsequently recrystallized in equal weight of urea and worked-up similarly as previously described. Each successive UC fraction was again recrystallized.

F. Analysis of CLA by GLC

CLA (1-2 mg) was methylated with 14% BF_3-MeOH (1 mL) and hexane (2 mL) in a screw cap tube (9 mL) with teflon cap for 30 minutes at room temperature with shaking. The reaction was halted with the addition of ddH_2O (1 mL). Phases were separated by centifugation at 300 x g for 10 min. The upper hexane phase was extracted and analyzed by GLC as described in chapter 3.

3. Results and Discussion

Figure 4-2 Counter Current Enrichment Scheme of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2



CLA isomers partition into the urea crystal (UC) adduct or mother liquor fraction (ML). CLA was recovered from the UC and ML fractions and recrystallized in the sequence illustrated by the above scheme. Fractions are labeled according to sequence of fractions from which it was derived starting from the original ML and UC fraction. The Δ9*c*,11*t*-18:2 isomer preferentially partitions into UC fractions while the Δ10*t*,12*c*-18:2 isomer partitions into the ML fraction. Consequently, the UC UC UC and ML ML ML ML fractions are most enriched in the Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 isomers, respectively.

Table 4-1 Composition of CLA Mixtures After Successive Equal Weight Urea Crystallizations

		1st Crystallization		2nd Crystallization				3rd Crystallization								4th Crystallization	
	Initial Composition	ML[1]	UC[2]	ML ML	ML UC	UC ML	UC UC	ML ML ML	ML ML UC	ML UC ML	ML[3] UC UC	UC ML ML	UC ML UC	UC UC ML	UC UC UC	ML ML ML	ML ML UC
% CLA[4]	93.5	95.6	91.0	96.0	95.6	94.5	89.5	94.8	96.3	97.4	92.2	96.5	90.3	90.3	79.3	93.2	95.9
% Δ9*c*,11*t*-18:2[4]	45.6	44.6	50.4	42.5	46.8	51.4	50.8	42.2	45.1	43.2	55.6	48.0	58.2	53.5	42.5	40.1	46.3
% Δ10*t*,12*c*-18:2[4]	50.3	52.6	42.4	55.3	50.2	46.2	37.4	53.6	53.1	54.6	40.6	48.4	34.6	36.9	17.4	57.9	51.3
% Δ11*c*,13*t*-18:2[4]	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0	0.2	0.1	0.1	0.3	0.1	0.1	0.1	0.2
cis / *cis* CLA[5]	0.8	0.8	2.6	0.5	1.5	1.3	3.1	0.6	0.3	0.6	1.5	0.9	4.0	5.0	18.7	0.3	0.8
trans / *trans* CLA[5]	3.1	1.9	4.4	1.6	1.4	1.1	8.6	3.4	1.5	1.4	2.1	2.5	2.9	4.4	21.3	1.6	1.4
9,11 : 10,12[6]	0.9	0.8	1.2	0.8	0.9	1.1	1.4	0.8	0.8	0.8	1.4	1.0	1.7	1.5	2.4	0.7	0.9
10,12 : 9,11[6]	1.1	1.2	0.8	1.3	1.1	0.9	0.7	1.3	1.2	1.3	0.7	1.0	0.6	0.7	0.4	1.4	1.1

[1]ML refers to the mother liquor fraction collected after filtration

[2]UC refers to the urea crystal fraction collected after filtration

[3]Fractions are identified by the lineage of crystallizations from which it was derived. i.e. ML UC UC and ML UC ML are derived from the 1st ML fraction, which was recrystallized, then the UC fraction recovered was again recrystallized producing the fractions ML UC UC and ML UC ML.

[4]Values are expressed as a percentage of total fatty acids by GLC analysis

[5]Values are expressed relative to total CLA fatty acids

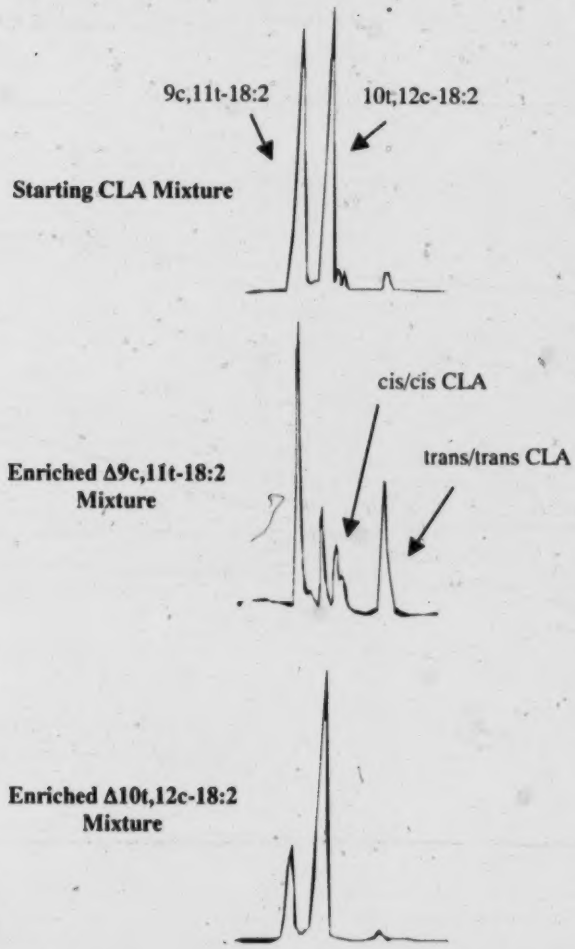
[6]Ratio of Δ9*c*,11*t*- to Δ10*t*,12*c*-18:2 or vice versa

Table 4-2 Composition of CLA Mixtures after Crystallization with 2.5 Fold Greater Urea

	Initial Composition	1st Crystallization	
Fraction		2.5 ML	2.5 UC
% CLA	93.5	94.4	87.4
% Δ9*c*,11*t*-18:2	45.6	29.7	50.4
% Δ10*t*,12*c*-18:2	50.3	69.1	44.1
% Δ11*c*,13*t*-18:2	0.2	0.1	0.5
cis / *cis* CLA	0.8	0.3	2.3
trans / *trans* CLA	3.1	0.8	2.7
9,11:10,12	0.9	0.4	1.1
10,12:9,11	1.1	2.3	0.9

These fractions were recovered after a mixture of CLA isomers were crystallized with 2.5 fold greater urea.

Figure 4-3 Chromatography of Mixtures of CLA



GLC chromatograms of a typical mixture of CLA from alkali isomerized LA. Counter current urea crystallization of the starting mixture yields one fraction enriched with the Δ9*c*,11*t*-18:2 and the other enriched in Δ10*t*,12*c*-18:2.

Initial crystallization of CLA with urea resulted in an enrichment of Δ9*c*,11*t*-18:2 in the UC fraction and enrichment of Δ10*t*,12*c*-18:2 in the ML fraction. The apparent preferential occlusion of the Δ9*c*,11*t*-18:2 isomer, may be attributed to the more overall linear geometry of its methyl tail compared to the Δ10*t*,12*c*-18:2 isomer. Assuming that the trans double bond of Δ9*c*,11*t*-18:2 is essentially linear, the methyl tail adopts a linear configuration between carbons 10 to 18. Comparatively, the cis double bond of Δ10*t*,12*c*-

18:2 kinks the fatty acid at carbon 12, therefore the methyl tail is only linear between carbons 13 to 18.

Figure 4-2 illustrates the systematic counter-current approach of fractionating the Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 isomers. In general, three types of fractions evolve. (1) Crystallization, recovery and subsequent recrystallization of only the UC or ML fractions produces highly enriched mixtures of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2, respectively. (2) Intermediary fractions can be further recrystallized and purified, but only those fractions with the greatest number of successive crystallizations of the same fraction will continue to be useful i.e "ML UC UC" and "UC ML ML". (3) Fractions derived from non-successive recrystallization of UC and ML fractions are of poorest enrichment, which can be pooled to begin the process anew.

It should be noted that although the "UC UC UC" fraction is greatly enriched in Δ9*c*,11*t*-18:2 compared to the Δ10*t*,12*c*-18:2 isomer, it also becomes enriched in cis/cis and trans/trans CLA isomers and other fatty acids that may also be occluded by urea (Figure 4-3) (Table 4-1, 4-2). The apparent enrichment of cis/cis and trans/trans isomers may be due in part to the loss of Δ10*t*,12*c*-18:2, resulting in a relative increase in proportion of these isomers.

The ratio of urea to CLA is also a factor affecting the level of isomer enrichment and weight yield between the UC and ML fractions. Comparatively, crystallization on an equal weight basis of urea and a mixture of CLA required 3 successive crystallizations to affect a 2.4 fold enrichment of Δ9*c*,11*t*-18:2 relative to Δ10*t*,12*c*-18:2 in the UC fraction, and only a 1.3 fold enrichment of Δ10*t*,12*c*-18:2 was found in the ML fraction. In a separate single trial experiment it was found that using a ratio of 2.5 g urea / CLA resulted in a 2.3 fold enrichment of the Δ10*t*,12*c*-18:2 after a single crystallization. The ratio of urea also affects the weight distribution of CLA recovered in the UC and ML fractions. When CLA was crystallized on an equal weight basis with urea, approximately 2-3 fold higher proportion of CLA was recovered in the ML fraction and was reversed when CLA was crystallized with 2.5 fold greater urea.

Overall, this method describes a simple and methodical approach to the selective enrichment of the Δ9*c*,11*t*- or Δ10*t*,12*c*-18:2 isomers. The counter-current method

described can be readily scaled up to produce large quantities of enriched isomers owing to its simplicity in procedure and use of common reagents. Using these enriched mixtures, it is then feasible to conduct human or large animal studies to examine the effect of Δ9*c*,11*t*- or Δ10*t*,12*c*-18:2 on health.

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Chapter 5 - CLA in Canadian Dairy and Beef Products

1. Introduction

CLA is a dietary fat that has beneficial effects for decreasing the risk of disease associated with cancer [1-4], atherosclerosis [5; 6], diabetes [7], lupus [5] and improved nutrient metabolism [8; 9]. Based on animal studies a level of 3 g CLA/day is equivalent in human diets [10]. There is no human data to support this claim conclusively. To begin to make recommendations, usual intake levels must first be determined, which requires data on CLA composition and content in the food supply. Intake levels in humans that confer these effects are unknown.

CLA is produced naturally in ruminant animals such as cattle, sheep and goats. The ruminant microorganism, *Butyrivibrio fibrisolvens* is responsible for the synthesis of the Δ9*c*,11*t*-18:2 isomer as an intermediate in the biohydrogenation of LA to vaccenic acid [11]. The content of Δ9*c*,11*t*-18:2 in dairy and beef products have been previously reported [12] and interestingly others have reported the presence of additional positional and geometrical isomers of Δ9,11-18:2, and Δ10,12-18:2 [13-15].

Current compositional information of CLA in foods is limited and has only been conducted in a few countries, not including Canada. Therefore, it is of interest to assess the content of CLA isomers in commonly consumed Canadian foods. Measurement of CLA content in Canadian dairy and beef products will be a valuable reference for health professionals.

2. Materials and Methods

A. Food Samples

A total of 20 different dairy foods consisting of milk, cheese and processed products and 5 beef cuts were obtained locally (Edmonton, Alberta, Canada). When possible, sampling was done from each of 4 similar products from different manufacturers. All other chemical reagents were obtained from BDH (BDH Inc.,

Toronto, Ontario, Canada) and Sigma (Sigma-Aldrich Canada, LTD., Mississauga, Ontario, Canada)

B. Lipid Extraction

Samples were analyzed from 2 or 5 g samples with the exception of butter, skim milk powder and milk where 0.5 g, 25 g and 5 mL quantities were used, respectively. Beef products were analyzed as both raw and cooked preparations from 10 g samples. The consistency of the cooked beef samples was monitored by measuring internal temperatures at the outer edge and inner portion of the sample. Lipid material was extracted in duplicate by the method of Folch et al. [16]. All lipid extracts were reconstituted in chloroform and stored in -70°C. Fat content was determined from dry lipid weights.

C. Saponification and Methylation of Lipid Extracts for GLC Analysis

In a screw cap tube, 5 mg of sample and 25 μg C19:0 of free fatty acid standard were saponified in NaOH-MeOH (0.5 M, 2 mL). Samples were heated for 1 hr at 110°C in a sand bath and then cooled. Samples were methylated according to the method of Werner et al. [17]. Hexane (2 mL) and 14% BF_3-MeOH (2 mL) were added to each sample and incubated at room temperature for 30 min with shaking. ddH_2O (1 mL) was added immediately and samples were vortexed briefly, then centrifuged at 300 x g for 10 min. The upper hexane phase was collected and the lower phase was re-extracted with hexane (2 mL). Total CLA content was quantitated by internal standard and expressed relative to the dried weight of lipid. Samples were then analyzed by GLC as described in Chapter 3.

3. Statistical Analysis

Orthogonal comparisons were used to compare the CLA content amongst dairy and beef products. The relationship between the level of fat and CLA was analyzed by Spearman ranked correlation analysis. A possible producer difference was analyzed by

two-way ANOVA. Differences between cooked and raw meats were analyzed by paired t-test. All analyses were done using SAS version 6.11.

4. Results

A. CLA Content in Commercial Dairy and Beef Foods

Total CLA content was determined by GLC analysis. Only the Δ9*c*,11*t*-18:2 isomer was detected in all foods analyzed. The level of CLA in dairy and beef foods ranged between 1.2-6.2 mg CLA/g fat, 0.001-4.3 mg CLA/g or mL sample or 0.03-81.0 mg CLA per usual serving size (Tables 5-1, 5-2). The levels of CLA when evaluated on a relative basis do not vary greatly. Orthogonal comparisons were used to statistically analyze the assorted samples used in this study. Samples were compared in the following groupings, dairy vs. beef, milk vs. non-milk, cheeses vs. all other dairies, and cooked vs. raw beef. To assess relative and absolute abundance of CLA, levels were expressed relative to fat content (mg/g fat), unit sample (mg/g or mg/mL) and per usual serving size. There was no significant difference in levels of CLA in the various foods relative to fat content, which was found to vary between, 1.2-6.2 mg/g fat. Levels of CLA were significantly different when expressed relative to sample size, which varied between 0.001-4.3 mg/g or mg/mL of sample. Differences were observed in the milk vs. non-milk ($p=0.001$) and cheese vs. all other dairies ($p=0.005$). Differences were also observed when levels of CLA were expressed per usual serving size, which varied between 0.03-81.0 mg/serving. Levels were significantly different for dairy vs. beef ($p=0.0001$), milk vs. non-milk ($p=0.0001$) and cheese vs. all other dairies ($p=0.0003$). The percentage of fat in each of these groupings was significantly different, dairy vs. beef ($p=0.008$), milk vs. non-milk ($p=0.0001$), cheese vs. all other dairies ($p=0.0001$) and cooked vs. raw beef ($p=0.005$). Although there were significant differences in the % fat content, there was no difference observed in the level of CLA relative to the level of fat. Therefore, the abundance of CLA in each of the foods is a function of total fat content. This conclusion was confirmed by Spearman ranked correlation between the % fat and level of CLA. The level of CLA expressed relative to fat content (mg/g fat) showed no correlation (0.08).

However, levels of CLA were correlated to % fat when levels of CLA were expressed per unit of sample (mg/g or mg/mL) (0.90) or per usual serving size (mg/serving) (0.58).

A possible brand difference amongst milk samples was analyzed by two-way ANOVA, amongst four producers of 1% milk, 2% milk and half/half cream (12% fat). No differences were found in the level of CLA amongst different producers. Significant differences were found amongst the three products, which differed in the level of fat content. No differences were observed relative to fat content, but differences were observed per unit of sample ($p=0.0001$) and per usual serving ($p=0.0001$). The level of fat was significantly different amongst the three types of products ($p=0.0001$). These results are similar to the analysis of the dairy products. Levels of CLA do not differ relative to the fat content, but are dependent on the absolute level of fat.

Split plot analysis of the raw and cooked meats show that there were significant differences amongst the types of meats ($p=0.006$). Therefore, pair-wise t-test comparisons were conducted for each type of meat. The level of fat was significantly different ($p<0.05$) between the raw and cooked state for all but the extra lean ground beef, which was probably due to loss of moisture during cooking. Only the sirloin roast had significantly more CLA after cooking ($p=0.02$) for levels of CLA expressed relative to the fat content. Both the rib ($p=0.005$) and sirloin tip roast ($p=0.05$) had significantly greater levels of CLA after cooking, when expressed per gram of sample. Again, differences in levels of CLA were observed mainly due to absolute differences in the level of fat.

5. Discussion

In the dairy and beef products analyzed, there is a single isomer of CLA in dairy and beef foods. Although other ruminant microorganisms may be able to synthesize other isomers [18], it appears that the majority of ruminant organisms either produce the one isomer, Δ9*c*,11*t*-18:2, or the specific organism, *Butyrivibrio fibrisolvens*, is the main producer of CLA. This suggests that the presence of multiple isomers in these foods would be unlikely. Thus, minor CLA isomers found by other investigators may be the

result of processing, or methylation procedures that cause intraisomerization or interisomerization.

Table 5-1 Levels of CLA in Dairy Products

	% Fat	mg / g fat	mg / g sample	mg / serving	Serving size(g)
Milk and Cream					
Skim Milk Powder	0.1±0.01	1.8±0.2	0.001±0.0003	0.03±0.01	25
Whole Milk	3.2±0.2	3.4±0.2	0.1±0.002*	25.6±0.5	250mL
1% Milk	1.0±0.04	4.3±0.4	0.04±0.003*	10.5±0.8	250mL
2% Milk	2.1±0.1	5.0±0.3	0.1±0.004*	25.8±1.2	250mL
Half/Half Cream	12.1±0.2	5.5±0.4	0.7±0.05*	10.0±0.7	15mL
Cheese					
Goat Cheese	28.5±1.8	2.7±0.2	0.7±0.03	34.3±1.5	50
Brie Cheese	27.9±1.7	3.8±0.5	1.0±0.1	52.1±6.9	50
Italian Parmesan Cheese[1]	28.3±2.5	4.2±0.5	1.2±0.3	12.0±2.7	10
Mozzarella Cheese	24.9±2.5	4.6±0.2	1.1±0.2	57.1±8.3	50
Cheddar Cheese	34.6±2.4	4.2±0.6	1.4±0.1	71.7±7.4	50
Imperial Cheddar Cheese	33.0±0.9	4.7±0.2	1.5±0.05	76.4±2.3	50
Farmer Cheese	28.9±3.1	4.7±0.7	1.3±0.2	64.7±10.0	50
Processed Products					
Cream Cheese	33.8±0.9	2.7±0.2	0.9±0.06	13.8±0.9	15
Yogurt	5.4±0.6	4.4±1.1	0.2±0.1	42.7±12.5	175
Butter	91.1±3.1	4.7±1.9	4.3±1.8	64.1±26.5	15
Cheese Whiz	19.1±2.7	4.9±0.3	0.9±0.1	13.7±1.8	15
Sour Cream	12.6±0.3	5.0±1.4	0.6±0.2	9.2±2.4	15
Processed Parmesan Cheese	28.5±1.2	5.3±0.6	1.5±0.2	15.0±1.8	10
Cottage Cheese[2]	3.1±0.1	5.9±1.4	0.2±0.03	26.7±5.1	150
Processed Cheese	24.3±1.8	6.2±1.2	1.4±0.2	70.4±11.2	50

All products were obtained locally during the Canadian spring and summer months. Each product was assessed from n=4 brands of the same product or 4 of the same product from different locations. Only the Δ9c,11t-18:2 isomer was identified.
[1]n=2, [2]n=3
*mg CLA/mL sample

Table 5-2 Levels of CLA in Beef Products

	% Fat	mg / g fat	mg / g sample	mg / 100 g serving
Raw Meats				
Sirloin Tip Roast	4.4±0.6	1.2±0.4	0.1±0.01	5.0±1.4
Extra Lean Ground Beef	9.1±1.5	1.4±0.2	0.1±0.03	11.2±2.9
Ground Beef	26.0±1.7	1.6±0.1	0.4±0.02	41.8±2.0
Rib Roast	13.4±1.6	3.0±0.7	0.4±0.1	40.3±11.6
Cooked Meats				
Extra Lean Ground Beef	10.0±1.5	1.2±0.1	0.1±0.03	11.5±2.5
Ground Beef	20.4±1.2	1.8±0.2	0.4±0.03	36.2±2.7
Sirloin Tip Roast	9.2±2.0	2.8±0.4	0.3±0.1	28.7±11.2
Rib Roast	27.8±1.2	2.9±0.5	0.8±0.1	77.6±11.5
McDonalds Quarter Pounder	25.6±0.4	3.2±0.4	0.8±0.1	81.0±7.7

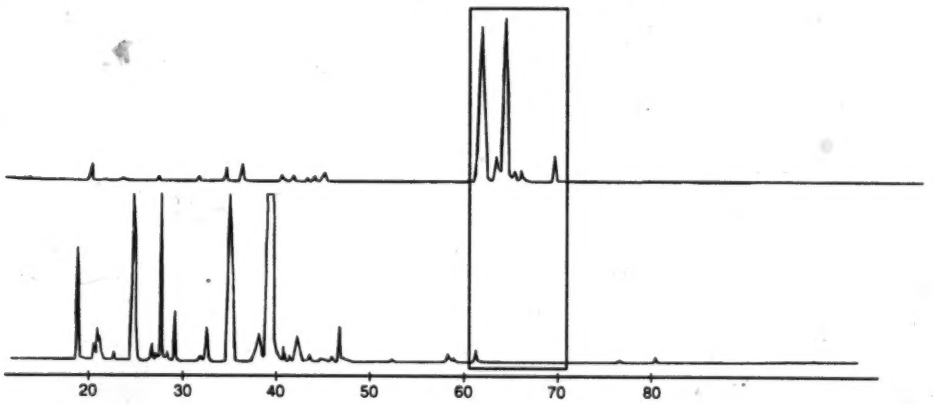
All products were obtained locally during the Canadian spring and summer months. Each product was assessed from n=4 brands of the same product or 4 of the same product from different locations.

Analytical issues have arisen in the literature to address the appropriateness of certain methods to derivatize CLA for GLC analysis. It is important to note that no single methylation procedure is adequate for the methylation of all lipid classes [19]. However, it should be emphasized that certain methods are more appropriate for use with a particular class of lipids than others. Analytical methods for the methylation of CLA have been reviewed by others. Methods employing acid catalysts such as HCl, BF_3, acetyl chloride and H_2SO_4 or base catalysts such as $NaOCH_3$, TMG, diazomethane have been assessed singly or in combination [14; 17; 19-21]. Some investigators recommend methylation using base catalysts because methods employing acid catalysts have been shown to produce unknown artifacts and cause intraisomerization.

Acid-catalyzed methylation using BF_3 was deemed suitable for use in this study. Typically, fatty acids are refluxed with BF_3-MeOH. It has been shown by Werner et al. [17] and Shantha et al. [20] that methylation of CLA with BF_3-MeOH at high temperature is responsible for artifact formation and intraisomerization. However, methylation at room temperature for 30 min or sustained periods up to 120 min reduces

and prevents artifact formation and intraisomerization [17]. Our assessments of this method in Chapter 3 also confirm that no artifacts are produced, and intraisomerization is not likely.

Figure 5-1 Typical GLC of a Food Sample and CLA Standard



Typical SP-2560 GLC chromatogram of a sample of beef (lower panel) and a CLA standard synthesized from safflower oil (upper panel). CLA peaks are enclosed within the boxed region. The beef sample contains a single peak corresponding to the Δ9*c*,11*t*-18:2 isomer.

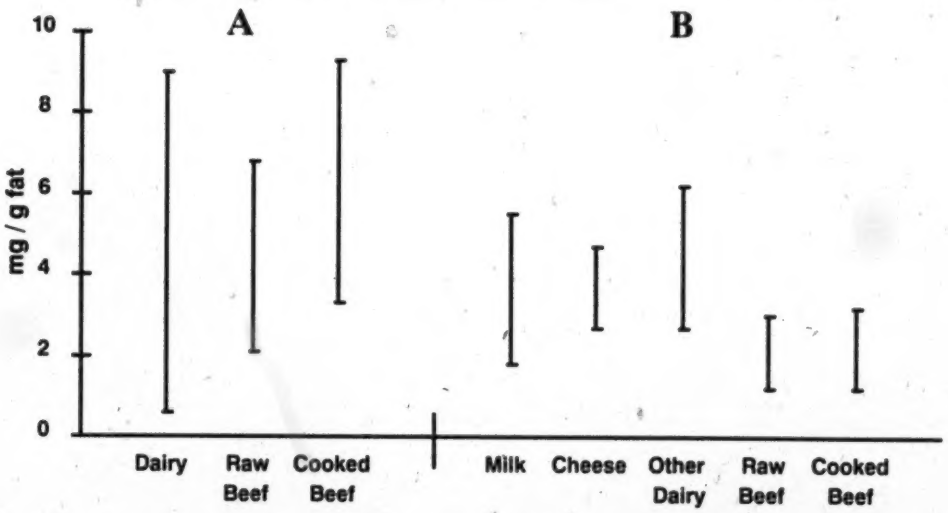
Room temperature methylation by Werner et al. [17] of cheeses showed the presence of multiple minor CLA isomers in addition to the abundant, Δ9*c*,11*t*-18:2 isomer. Although no differences in the level of isomers were observed, differences in the CLA isomer distribution was detected. These differences were attributed to different starter cultures, processing conditions and aging periods. Minor isomers of CLA were not detected in the foods analyzed in this study. The amount of CLA injected for GLC analyses in this study and that of Werner et al. [17] were comparable at nanogram amounts. This discrepancy may reflect different manufacturing processes which altered the CLA profile. Others have shown that manufacturing protocols do not result in the loss of CLA [22], but may actually enhance CLA content [23]. Food additives increase both total CLA and the level of the Δ9*c*,11*t*-18:2 isomer [24]. Atmospheric processing in the presence of oxygen at high temperature increases total CLA content, without an increase

in the Δ9*c*,11*t*-18:2 isomer [25]. Therefore, the presence of minor isomers may be due to various conditions and ingredients used to prepare those foods.

Cooking meat, such as ground beef has been suggested by Ha et al. [13] to increase the amount of total CLA. Of the four cuts of meats, only the sirloin roast cut was observed to contain an increased amount of CLA after cooking (1.2 mg/g fat to 2.8 mg/g fat, respectively). Shantha et al. [25] has shown that various cooking methods at different temperatures do not affect CLA content relative to per gram of fat. In general, this observation is consistent with our findings with the exception of the sirloin roast. No change in the level of CLA per gram of fat in ground beef was observed after cooking, which is not in agreement with the result observed by Ha et al. [13]. Perhaps, this difference may be due the length of the cooking. Cooking does significantly alter the concentration of CLA relative to the entire sample on a weight basis (mg/g sample). Both the sirloin tip and rib roasts had significantly greater levels of CLA after cooking, which coincided with an increase in fat content. This increase in fat content is most likely due to moisture loss, and is consistent with observations made by Shantha et al. [25], that were attributed to changes in the edible portion.

Levels of CLA detected in this study are lower than those reported by others (Figure 5-2) [13; 14; 17; 23; 25-28]. This may be in part attributed to differing seasonal feeding practices and dietary regimens. Diets containing cereals and maize tend to have low milk CLA [29]. Pasture and grass feeding practices with concentrates and roughage rich in polyunsaturated fatty acids tend to produce higher milk [29-32] and meat [27] CLA. Enhanced CLA content is also increased with oil supplementation with rapeseed, rich in LA [32]. Feeding frequency is also a factor as cows fed restricted amounts of similar diets contain higher milk CLA than ad libitum fed cows [31]. Overall, cows fed diets rich in grasses or polyunsaturated fatty acids of LA tend to yield higher amounts of CLA in both milk and muscle.

Figure 5-2 Levels of CLA (mg/g fat) Reported by Others and in this Study



Comparison of levels of CLA (mg/g fat) reported by others (A) to levels observed in this study (B). The ranges of values are reported for each category indicated. Values by others are compiled from Ha et al.(1989), Werner et al.(1992), Chin et al.(1992), Shantha et al.(1992;1994;1995), Lin et al.(1994).

Differences in seasonal feeding practices may not completely explain why low levels of CLA were detected. Foods were obtained during the spring and summer Canadian months, therefore, cows and cattle would likely be pasture fed and would be expected to have higher CLA content. Several other factors in addition to seasonal variation and diet may be responsible for the lower CLA content detected in the foods analyzed in this study. No differences in CLA content were observed in this study amongst different milk producers and products. Investigators analyzing raw milk note that there is high variability of CLA content amongst experimental animals [31], therefore animal genetics may also be a confounding factor [29; 32]. Parity has also been shown to influence the level of milk fat CLA [32].

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Chapter 6 – Effect of Enriched CLA Mixtures on MDA-MB-231 Cell Proliferation, Membrane Composition, and PGE_2 Synthesis

1. Introduction

The study of the anti-carcinogenic effects of CLA and the mechanism of action remains a hot topic of study, especially in the area of breast cancer. The focus of this chapter is to determine how Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 isomers modulate essential fatty acid metabolism in a mammary tumor cell line. The MDA-MB-231 mammary tumor cell line is characterized as an aggressively growing metastatic cell type that produces high levels of PGE_2 and is capable of producing tumors in nude mice [1; 2]. Therefore, effects of CLA on cell proliferation, membrane composition, and PGE_2 synthesis should be readily observed in this model system.

The majority of studies to date have utilized a mixture of CLA isomers containing predominantly Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 in equal amounts (Appendix A, Table A-1). Very little is known about the individual effects of these major isomers. Initial studies investigating the anti-carcinogenic effects of CLA show that all isomers are incorporated into triglycerides but Δ9*c*,11*t*-18:2 is preferentially incorporated into total membrane phospholipid [3; 4] which suggests that this isomer is responsible for anti-carcinogenic effects observed with the use of a mixture of CLA isomers.

Changes in membrane composition have implications for modulating essential fatty acid metabolism, and ultimately eicosanoid synthesis, a possible mechanism of CLA action. LA can be desaturated and elongated to AA, the substrate for the synthesis of eicosanoids. AA used for eicosanoid synthesis is released by the action of phospholipases from phospholipids. Eicosanoids are important molecules responsible for many cellular functions, reviewed in [5]. CLA may compete for incorporation with LA or AA into membrane phospholipid thus reducing incorporation of LA or AA [6]. CLA can be elongated and desaturated [7-9]. Thus another possibility is competition between LA and CLA for chain elongation and desaturation, consequently reducing the availability of AA in the membrane. Several studies have shown that CLA modulates eicosanoid synthesis and inhibits the production of PGE_2 produced by tumors [10; 11], immune cells [12; 13]

and bone [14-16]. Overall, the evidence implicates CLA in changes in essential fatty acid metabolism but it is not known how these changes occur and which isomer is responsible.

There is conflicting evidence in the literature supporting this mechanism of action in breast cancer models. Ip et al. [17; 18] showed that although CLA was incorporated into total membrane phospholipid of mammary tissue of rats, there was no alteration in level of LA or AA. In models other than breast cancer, it has been shown that CLA can affect essential fatty acid metabolism and is uniquely incorporated within different phospholipid fractions. Mice fed a mixture of CLA incorporated CLA into total liver phospholipid and significantly reduced LA and tended to decrease AA in the phospholipid fraction [7]. In a murine epidermal cell line, CLA supplemented alone is incorporated into membrane phospholipid fractions similar to LA [19], suggesting that CLA can replace LA [11]. Stangl et al. [20] show that in rats fed CLA there is a dose-dependent reduction of LA and AA into liver membrane phospholipid fractions of PC, PE, PI, and PS, however a mixture of CLA was used and the effects of individual isomers were not discernable.

There is growing evidence suggesting that the Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 isomers may have different and independent biological effects. Using milk highly enriched in Δ9*c*,11*t*-18:2, Ip et al. [21] showed that this preparation exerted anti-carcinogenic effects. The Δ10*t*,12*c*-18:2 but not the Δ9*c*,11*t*-18:2 isomer has been shown to improve body composition by enhancing lean body mass and reducing fat mass [22-24]. Although the Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 isomers have been shown to exert different biological effects, the underlying mechanisms have yet to be clearly delineated.

The mechanism of action by which CLA inhibits tumorigenesis in breast cancer has been an area of active investigation. Early studies suggested that CLA did not alter essential fatty acid metabolism and was not the mechanism of action by which tumor growth was inhibited in animal models of breast cancer. However, this conclusion was based on analysis of total phospholipid. Fatty acids in different membrane phospholipid fractions are not uniformly distributed. In particular, AA is enriched in PI and PC, which are specific pools of AA released by specific phospholipases for eicosanoid synthesis. Analysis of individual phospholipid fractions may provide stronger evidence showing an

effect of CLA on essential fatty acid metabolism. There is evidence by other investigators who have observed changes in essential fatty acid metabolism in both total and individual phospholipid fractions in non-breast cancer models [7; 20]. Typically, in animal diets, LA is present in far greater proportion relative to CLA, thus making it difficult to ascertain if CLA is competitively displacing LA and its product AA from membrane phospholipid. Conversely, in cell culture studies the effects of CLA is often observed under conditions in the absence of LA. The majority of studies to date have utilized a mixture of isomers, therefore the effects of individual isomers is not clear.

The objective of the present study is to examine the effect of enriched mixtures of Δ9*c*,11*t*- or Δ10*t*,12*c*-18:2 isomers on tumor growth. Changes in growth may be associated with effects on substrate availability of LA as a mechanism for inhibiting tumor growth. Modification of LA, the essential fatty acid precursor of AA has consequences for modulating PGE_2 synthesis and growth of tumor cells. Growth of MDA-MB-231 cells cultured in enriched mixtures of Δ9*c*,11*t*- or Δ10*t*,12*c*-18:2 will be assessed by radioactive incorporation of ^{3}H-thymidine. The competitive incorporation of Δ9*c*,11*t*- or Δ10*t*,12*c*-18:2 into membrane phospholipid fractions in the presence of an equal amount of LA will be examined. PGE_2 synthesis will be measured by GC-MS.

2. Materials and Methods

A. Fatty Acid Treatments and Cell Culture Conditions

Three different CLA mixtures were used, which include an (1) equal mixture of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2, (2) enriched mixture of Δ9*c*,11*t*-18:2 and (3) enriched mixture of Δ10*t*,12*c*-18:2. The equal mixture of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2, will be referred to as "Mix" in the remainder of this Chapter and reference to the enriched mixtures will be to the major isomer present, Δ9*c*,11*t*- or Δ10*t*,12*c*-18:2.

The preparation of these mixtures and their composition are described in Chapters 3 and 4. LA was extracted and purified from safflower oil, which is described in Chapter 3 and AA was purchased (Aldrich, Cat No. 23,384-6 Oakville, Ontario, Canada). All fatty acids were stored in ethanol at a concentration of 5 mg/mL at –25°C.

MDA-MB-231 mammary tumor cell lines were obtained from the American Tissue Culture Centre (ATCC). MDA-MB-231 cells are epithelial like and adherent in culture. Cells were cultured in Iscoves Modified Dulbeco Media (IMDM) (Gibco BRL, Cat No. 12200-036, Grand Island, NY, USA) containing fetal bovine serum (FBS, 5% v/v) (Gibco BRL, Cat No. 10437-028, Grand Island, NY, USA) and antibiotic-antimycotic (A/M, 1% v/v) (Gibco BRL, Cat No. 25200-056 Grand Island, NY, USA). Media was sterile filtered using a 0.22 μM bottle top filter (Corning Inc., Cat No. 430513, Corning, NY, USA). Cells were routinely passaged at a density of 0.5×10^6 cells in a 75 cm^2 vented flask every 4 days (Corning Inc., Corning, Cat No. 430641 NY, USA or Sarstedt Inc, Cat No. 83.1813.002, Newton, NC, USA). Cells were detached from the flasks by incubating with trypsin-EDTA (5 mL) (Gibco BRL, Cat No. 25200-056, Grand Island, NY, USA) for 10 min and then decanted into a sterile centrifuge tube (15 mL) (Fisher Scientific, Cat No. 05-539-1, Pittsburgh, PA, USA) and spun down at 300 x g for 10 min (Jouan, Canberra Packard, Mississauga, Canada). Cells were maintained in a 37°C incubator with a humidified atmosphere of CO_2 (5%) and air (95%). Prior to use, FBS was heat inactivated at 55°C in a water bath for 30 min, then aliquoted and stored in sterile tubes (10 mL).

The experimental fatty acid treatments used in proliferation experiments were prepared as follows. In the initial proliferation experiment using 1% (v/v) FBS, stock IMDM containing FBS (1% v/v) and A/M (1% v/v) was added to bovine serum albumin (BSA) (0.1% w/v) (Sigma, Cat No. A-7906, Lot No. 56H0659, Oakville, Ontario, Canada) and sterile filtered. This was added to fatty acids dried down under nitrogen and low temperature in sterile centrifuge tubes (15 mL). All fatty acids were added to obtain a final concentration of 60 μM, unless otherwise stated. Each tube was vortexed and then incubated at 37°C in a shaking water bath for 30 min.

In the subsequent proliferation experiment using 5% (v/v) FBS, FBS was not sterile filtered prior to use and added directly to the dried fatty acids. IMDM and BSA were sterile filtered prior to use.

B. Proliferation of MDA-MB-231 Cells Cultured in Media Containing 1% FBS

The effect of CLA on growth was assessed by incorporation of radioactive, ^{3}H-thymidine, as a marker of proliferation. For experiments, cells were grown to >70% confluency over a 4 day period. Media was decanted and trypsin-EDTA (5 mL) was added and then incubated for 5 min in the incubator. Cells were transferred to a centrifuge tube (15 mL). Flasks were washed with 5 mL of IMDM and pooled. Cells were spun down at 300 x g for 10 min. Media was decanted and cells were resuspended in 1 mL of media and counted using a hemocytometer with trypan blue dye for viability determination. MDA-MB-231 cells (2 x 10^{4} cells/well) were then seeded into sterile 24 well tissue culture treated polystyrene non-pyrogenic plates (Corning Inc., Cat No. 3524, Corning, NY, USA). Cells were cultured in 1 mL of media for 2 days. Media was then replaced with media supplemented with one of the following fatty acid treatments, (1) LA(60 μM), (2) LA(120), (3) LA+Mix, (4) LA+Δ9*c*,11*t*-18:2 or (5) LA+Δ10*t*,12*c*-18:2. (6) LA + Mix (10 μM) and (7) LA + Mix (30 μM). LA(60) was the control treatment used as a baseline reference at a level present in all the CLA treatments. LA (120 μM) was the control treatment to account for any effects associated with the total amount of exogenous fatty acid used. The LA + Mix (10 μM) and LA + Mix (30 μM) treatments were used to examine a dose response effect of CLA.

Each lipid treatment was applied to quadruplicate wells per plate and incubated for 2 days. On day 4, cells were pulsed with ^{3}H-thymidine ([methyl-^{3}H] thymidine in aqueous solution) (50 μL, 5 μCi, 85 Ci/mmol)(Amersham Pharmacia Biotech Inc., Quebec Canada) and incubated for 4 hrs. The media was discarded and trypsin-EDTA (300 μL) was added to each well and incubated for 5 min in the incubator to allow cells to detach, then phosphate buffered saline (PBS) (600 μL, pH 7.4) was added and cells were resuspended. Triplicate aliquots (200 μL) from each well were transferred to a 96 well plate and harvested using a multiwell harvester (Skatron, Lier, Norway) onto glass fibre filter mats (Skatron, Suffolk, United Kingdom) then dried overnight. Cut outs from the mats were transferred to scintillation vials (4 mL) to which 4 mL Ecolite (ICN, Cat No. 882475, CA, USA) was added and counted for radioactivity (Beckman 5000, Beckman Instruments, Palo Alto, CA, USA).

C. Proliferation of MDA-MB-231 Cells Cultured in Media Containing 5% FBS

The proliferation assay was done as described in the previous section with minor modifications. Although FBS was purchased sterile, it was filtered before use. Analysis of FBS showed that the fatty acid composition was changed by filtering (Appendix B) and possibly other factors affecting growth, therefore filtering was discontinued. Stock IMDM was prepared only with A/M (1% v/v) and then sterile filtered. The FBS was added to the media at the time of passaging and for experimental use.

D. Incorporation of CLA Treatments into Membrane Phospholipid

MDA-MB-231 cells were seeded at a density of 1 x 10^6 cells into 6 x 75 cm^2 vented flasks and cultured in stock IMDM (9.5 mL) to which was added FBS (0.5 mL, 5% v/v). The following 6 treatments were prepared, (1) control (Ctrl, no exogenous fatty acid), (2) LA (60 μM), (3) LA(120), (4) LA+Mix, (5) LA+Δ9*c*,11*t*-18:2 and (6) LA+Δ10*t*,12*c*-18:2. The experimental lipid treatments (10 mL) were prepared in the same way as described in the section C. Cells were grown for 2 days at which time the media was exchanged with one of the experimental fatty acid treatments and incubated for an additional 2 days. Cells were harvested by incubating with trypsin-EDTA (5 mL) for 5 min, then decanted into a centrifuge tube (15 mL). The flask was rinsed with IMDM (5 mL) and pooled. Cells were then spun down at 300 x g for 10 min. The media was decanted and the cells were washed with PBS (5 mL) and then spun down again at 300 x g for 10 min. The PBS was discarded leaving behind a clear pellet of cells for fatty acid analysis.

E. Incorporation of CLA into MDA-MB-231 Membrane Phospholipids Over 48 hrs

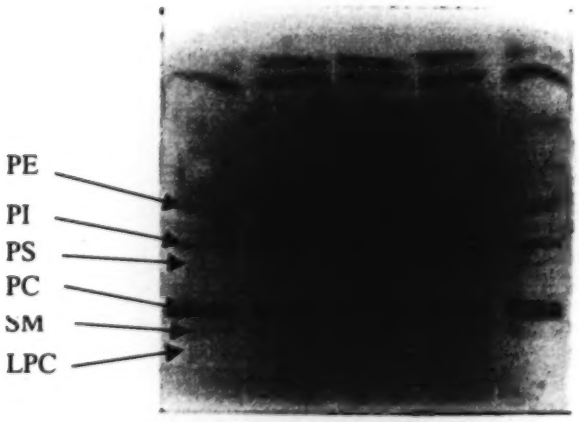
Incorporation of CLA mixtures was assessed over time at 5, 24 and 48 hrs. The following three treatments, (1) LA(60 μM)+Mix, (2) LA+Δ9*c*,11*t*-18:2 and (2) LA+Δ10*t*,12*c*-18:2 were prepared as described in section C. For each treatment and time point, three flasks of cells were used. Cells were allowed to adhere and grow for 2 days

before the media was exchanged for the experimental fatty acid treatments. At 5, 24 and 48 hours, cells were collected and processed for fatty acid analysis.

F. Extraction and TLC Separation of Membrane Phospholipid Fractions

Cells were resuspended in KCl (1.6 mL, 0.1 M) and transferred to a glass screw cap tube with a teflon lined cap (15 mL). The centrifuge tube was washed with MeOH (0.8 mL), then pooled and vortexed. Chloroform : methanol (1:1, 2.0 mL) was added followed by chloroform (2.7 mL), and chloroform : methanol (2:1, 2.5 mL) with vortexing between additions. The mixture was separated over-night into an upper aqueous and lower organic phase in a cold-room (5°C). The lower chloroform phase was transferred into a disposable culture tube (Fisher Scientific, Cat No. 14-961-27, 13 x 100 mm, Pittsburgh, PA, USA). The upper phase was re-extracted with chloroform (5.36 mL) and pooled. The two phases were separated by centrifuging at 300 x g for 10 min (Sorvall SS-3 Superspeed Centrifuge, Norwalk, Connecticut, USA). The chloroform was evaporated under a gentle stream of nitrogen with mild heating.

Figure 6-1 Typical H-plate Separation of Membrane Phospholipid Classes



Typical H-plate separation of phospholipid classes extracted from MDA-MB-231 cells. The bands are identified as follows phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin (SM) and lysophosphatidylcholine (LPC). LPC is not seen in the samples, but its expected position is indicated.

Phospholipid classes were separated by TLC. H-plates (20 x 20 cm, 250 microns) (Analtech, Inc. Cat No. 10011, Newark, DE, USA) were washed in a TLC tank with hexane, heat inactivated at 100°C for 1 hr, then 2 cm lanes were scored. A TLC tank lined with filter paper was saturated with chloroform : MeOH : 2-propanol : KCl 0.25% (w/v) : triethylamine (30 : 9 : 25 : 6 : 18) for at least 30 min before use. Lipid samples in the disposable glass tubes were reconstituted in chloroform : MeOH (1:1, 100 μL), then applied using a Hamilton syringe (100 μL, Hamilton Company, Reno, Nevada, USA). The glass tube was washed and re-applied in the same manner. Phospholipid standards of PC, PS, PI and PE (Sigma, Oakville, Ontario, Canada) were applied to a separate lane for identification purposes. Plates ran until the solvent front migrated to the top of the plate. Plates were then air dried in a fume hood then sprayed with 8-anilino-1-naphthalene sulfonic acid (0.1% w/v). Bands were visualized under UV light (Figure 6-1) and identified by comparison to co-migrating standards. Bands were then scored and scraped onto weighing paper and transferred to glass screw cap tubes with a teflon lined cap (15 mL).

G. Methylation of Phospholipids, GLC and GC-MS Analysis

Lipids were methylated on silica in 15 mL glass screw cap tubes in hexane (1 mL) and 0.5 M NaOMe-MeOH (2 mL) (Aldrich, Oakville, Ontario, Canada). Samples were heated in an oven at 50°C for 15 min and the reaction was terminated with the addition of glacial acetic acid (0.1 mL). Hexane (1 mL) and ddH_2O (5 mL) were then added. The mixture was spun down at 300 x g to separate phases. The upper hexane phase was transferred to a 2 mL GC vial and dried under a gentle stream of nitrogen with mild heating. The lower phase was re-extracted with hexane (2 mL) and pooled. The methylated lipid was reconstituted in hexane (30-50 μL) for GLC analysis.

GLC analysis was performed similarly as described in Chapter 5. Samples were also analyzed by GC-MS on an HP5890 GC coupled to a HP5970 MS detector. Methyl esters were separated on a BPX70 fused silica capillary column (30 m, 0.25 micron film thickness, 0.25 mm I.D.) (SGE, Part No. 054622, Australia; Local Distributor: Rose Scientific Ltd., Edmonton, Alberta, Canada) The injector and detector ports were set at

250°C and 280°C, respectively. Methyl esters were eluted using a temperature program set initially at 120°C, then increased at 2.5°C/min and held at 164°C for 4 min, then increased at 2.5°C/min and held at 205°C for 4 min, then increased at 3°C/min to 260°C to complete the run. The carrier gas was helium set to flow at 2 mL/min. Samples were injected in split mode with a 10:1 split. Diagnostic ions were used to identify C18:3 and C20:4 fatty acids. Peaks were screened for mass 79, which is characteristic of polyunsaturated fatty acids containing 3 or more double bonds. Characteristic masses used to identify C18:3 fatty acids were 292.4 (M) and 261 (M-31). The masses used to identify C20:4 were 318.5 (M), 287.5 (M-31), 244.5 (M-74), and 202.5 (M-116) [25].

H. Cell Culture Conditions, CLA Treatments, and Extraction of PGE_2

Experimental fatty acid treatments were prepared in the same manner as described in section C, with slight modifications. The BSA was no longer filtered prior to use and all fatty acid treatments included AA. MDA-MB-231 cells (2 x 10^6 cells) were seeded into 162 cm^2 vented flasks (Corning Inc., Costar, Cat No. 3151, Corning, NY, USA) and grown for 3 days in IMDM (15 mL) containing 5% FBS. The following fatty acid treatments were prepared, (1) AA (60 μM), (2) AA+LA(60), (3) AA+LA(120), (4) AA+LA+CLA (5) AA+LA+Δ9*c*,11*t*-18:2 and, (6) AA+LA+Δ10*t*,12*c*-18:2. Fatty acid treatments were prepared in a final volume 15 mL. After replacing the media with the fatty acid supplemented media, cells were stimulated with a calcium ionophore, A23187 (10 μM) and phorbol ester, phorbol-12-myristate-13 acetate (PMA) (400 nM) (ICN Biomedicals Inc, Ohio, USA). Cells were then incubated for 24 hrs, at which time, the media was decanted into a 15 mL centrifuge tube (Fisher Scientific, Cat No. 05-539-1, Pittsburg, PA, USA) and spun down at 300 x g for 10 min to pellet any cells present. Cells were counted for viability by trypan blue exclusion under a light microscope. Media was transferred to a 20 mL glass screw cap tube, with a teflon lined cap to which was added an internal standard, 11-deoxy-PGE_1 (1 μg) (Cayman Chemical, Cat No. 37786-00-8, Ann Arbor, MI, USA). The media was acidified to pH 3.0-3.5 using HCl (0.5 N). Eicosanoids were extracted with ethyl acetate (2 x 10 mL). The mixture of ethyl acetate and media was spun down at 300 x g for 10 min to separate phases. The ethyl acetate

washings were transferred to 15 mL glass screw cap tube and evaporated to dryness under nitrogen with mild heating. The remaining cells in the flask were detached using trypsin-EDTA (7 mL) then counted and viability was determined using trypan blue exclusion.

I. Derivatization of PGE_2 for GC-MS Analysis

Prostaglandins were methylated according to the method of Yurawecz et al. [26] with slight modifications. Typically, free fatty acids were dissolved in tetrahydrofuran / methanol (4:1, 1mL) to which was added 10% trimethylsilyldiazomethane (TMSD) in hexane (0.5 mL) (Aldrich, Cat No. 36,283-2 Oakville, Ontario, Canada). The reaction was incubate for 30 min at room temperature with occasional shaking. The reaction was terminated with 5 drops of glacial acetic acid with gentle shaking to destroy excess yellow TMSD (allow 1 min to let color fade). Methyl esters were extracted with isooctane (2 x 3 mL) after the addition of ddH_2O (5 mL). Isooctane was transferred to a clean 15 mL glass screw cap tube with a teflon lined cap and evaporated to dryness under nitrogen and mild heating.

Prostaglandin methyl esters were converted to their methoxyamine derivatives according to the method of Maclouf and Rigaul [27] with minor modifications. Typically, methyl esters are dissolved in stock solution of saturated O-methylhydroxylamine HCl (Sigma, Cat No. M113-9 Oakville, Ontario, Canada) (100 µL) (0.1 g in 0.5 mL anhydrous pyridine) (Aldrich, Cat No. P-3776 Oakville, Ontario, Canada). The reaction is heated for 1 hr at 40°C in an oven. Then, excess pyridine is evaporated until a white precipitate is produced.

Hydroxyl groups on eicosanoids were converted to more stable t-butyldimethylsilyl ether derivatives for GC-MS analysis according to the method of Parsons et al. [28] with slight modifications. Methyl esters were dissolved in N,N-dimethylformamide (500 µL) (Sigma, Cat No. D-8654, Oakville, Ontario, Canada) to which was added 500 µL of a 1 mmol solution of t-butyldimethylchlorosilane (Aldrich, Cat No. 19,050-01, Oakville, Ontario, Canada) (0.1507 g) and 2.5 mmol imidazole (Sigma, Cat No. 2-2399, Oakville, Ontario, Canada) (0.1702 g) in N,N-

dimethylformamide (1 mL). The reaction was heated at 80°C for 90 min. After the reaction cooled, ddH_2O (5 mL) was added and the eicosanoid derivative was extracted with hexane (2 x 2 mL). The aqueous and hexane phases were separated by spinning at 300 x g for 10 min.

GC-MS conditions are the same as those described in section G. In addition to PGE_2, the eicosanoid of primary interest, the presence of leukotrienes was also assessed. The literature reports that the leukotrienes, LTB_4, 5-, 12-, and 15-hydroxyeicosatetraenoic (HETE) acids are also produced by the MDA-MB-231 [29; 30]. Eicosanoids were identified by comparison to authentic standards or fragmentation patterns reported in the literature [31-33]. Only PGE_2, LTB_4, and 5-HETE were obtained commercially (Cayman Chemical, Ann Arbor, MI, USA). The prominent ions used to detect prostaglandins and leukotrienes are listed in Table 6-1. The production of possible new leukotrienes derived from CLA was also monitored. The diagnostic ion used for this purpose was m/z = 73, the fragment arising from the first 2 carbons from the carbonyl carbon, a feature present in the other eicosanoids.

Table 6-1 Prominent Ions of Eicosanoids Detected by GC-MS

Molecule	Prominent ions
PGE_2	566, 337, 267, 73
11-deoxy-PGE_1	438, 406, 374, 73
LTB_4	467, 335, 245, 73
5-HETE	391, 245, 73
12-HETE	391, 337, 255, 73
15-HETE	391, 267, 215, 73

3. Statistical Analysis

The replication indicated refers to treatments applied to different passages of cells. Effects of CLA on proliferation in 1% FBS (n=6-10), proliferation in 5% FBS (n=5), fatty acid composition within a phospholipid fraction (n=4-5), and PGE_2 synthesis (n=6-7), were analyzed by 1-way ANOVA, blocked by passage number. An index of LA to

AA conversion was calculated as the ratio of % AA to % LA of total fatty acids based on the fatty acid compositional data (Tables 6-2, 6-3, 6-4 and 6-5) and also analyzed by 1-way ANOVA, blocked by passage number.

The fatty acid compositional data assessing the effect of CLA over time was analyzed by 2-way blocked ANOVA with main effects of time and CLA treatment, blocked by passage number (n=3). Fatty acids having significant model effects were reanalyzed by 1-way ANOVA for effects on time within each CLA treatment, and effects of CLA treatment at each given time. The relative levels of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 in each phospholipid fraction at each time was analyzed by 1-way ANOVA blocked by passage number. An index of LA to AA conversion was calculated as the ratio of % AA to % LA of total fatty acids at each time point was also determined and analyzed by 1-way ANOVA, blocked by passage number.

All analyses were done using the SAS version 8 statistical package. Duncans multiple range comparisons were performed with balanced data and when required the Pdiff function was used for unbalanced data.

4. Results

A. Preliminary Investigations and Rationale for Fatty Acid Treatments

Preliminary work (Appendix C) testing the effect of CLA preparations in the absence of LA showed that the Δ9*c*,11*t*-18:2 isomer inhibited growth of MDA-MB-231 cells to a greater extent than Δ10*t*,12*c*-18:2 or Mix treatments. All CLA treatments appeared to stimulate growth relative to cells grown only in 1% FBS. The level of 1% FBS may be sub-optimal for growth and addition of any exogenous fatty acids may appear to enhance growth. The absence of LA more likely represents a model of essential fatty acid deficiency, and CLA make evoke an unphysiological response, which it may not make when LA is also present. This was observed in a preliminary experiment showing that CLA readily incorporated into membrane phospholipid fractions, but this incorporation is reduced in the presence of LA (Appendix D, Figures D-1 and D-2). Delivery of fatty acids bound to BSA was also determined to be a requirement showing that fatty acids delivered in the absence of BSA inhibited proliferation with the same

treatments (Appendix C). Binding of fatty acids to BSA was confirmed by co-elution of ^{14}C-linoleic acid with BSA on packed columns (Appendix E, Figures E-1 and E-2).

B. Effect of CLA on Proliferation when Cultured with 1% FBS

The CLA treatments did not profoundly inhibit proliferation of MDA-MB-231 cells. All Mix treatments, LA+Mix, LA+Mix(10) and LA+Mix(30), significantly inhibited proliferation relative to LA(120) but not LA(60). There was no dose dependent inhibitory response with the Mix treatments. The Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 treatments significantly inhibited proliferation relative to LA(60) and LA(120) (Figure 6-2).

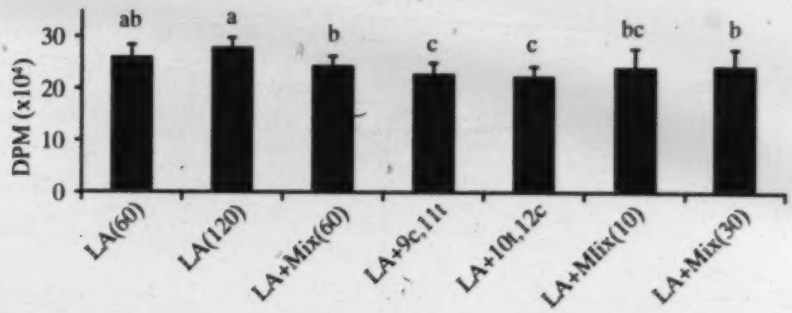
C. Effect of CLA on Proliferation when Cultured with 5% FBS

The inhibitory effect of the different CLA mixtures was not very pronounced under growth conditions containing lipid treatments in media containing 1% FBS. These conditions may not have been conducive to overall growth, which may have been a greater contributing factor to growth relative to any growth inhibitory effects due to CLA. The experiment was repeated in which CLA treatments were added to media containing 5% FBS (Figure 6-3). To test whether CLA inhibits proliferation relative to LA a separate experiment was first conducted to verify that LA enhances tumor growth in culture containing 5% FBS. Increasing levels of LA added to 5% FBS enhanced growth when measured by cell counts (Appendix F). Growth was also shown not be enhanced with human serum (Appendix G).

The greatest amount of cell growth of MDA-MB-231 cells occurred in cells treated with the control treatments, LA (60 and 120μM) (Figure 6-3). The Mix treatment did not significantly inhibit growth relative to the control treatments, but was intermediate relative to the Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 treatments. The Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 treatments significantly inhibited growth relative to the controls. At lower concentrations of Mix treatment, there was inhibition of growth but no dose response. The absolute decays per minute (DPM) were much higher in cells treated with 1% FBS (Figure 6-2) compared to cells treated with 5% FBS (Figure 6-3), which suggests that the cells grew better in 1% FBS. This discrepancy is most likely attributed to subtle changes

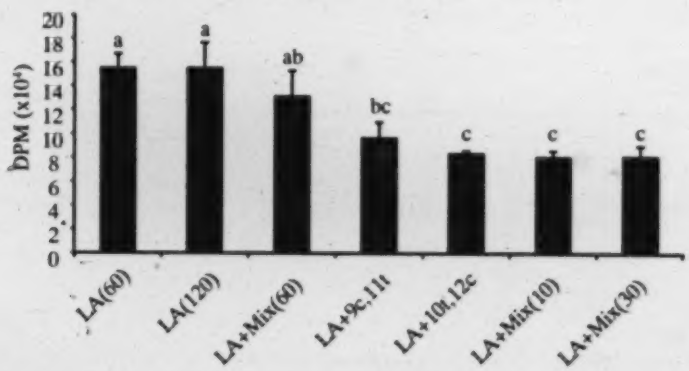
between these separate experiments, such as the collection of cells and washing of excess ^{3}H-thymidine.

Figure 6-2 Effect of CLA Mixtures in the Presence of LA on Proliferation of MDA-MB-231 Cells when Cultured in Media Containing 1% FBS



Proliferation was quantified as a measure of radioactive incorporation of ^{3}H-thymidine expressed in decays per minute (DPM). Values are mean ± SEM (n=6-10). 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal mixture of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2. All fatty acids were provided at a concentration of 60 μM, unless otherwise indicated. Treatments with different letters are significantly different ($p<0.05$).

Figure 6-3 Effect of CLA Mixtures in the Presence of LA on Proliferation of MDA-MB-231 Cells when Cultured in Media Containing 5% FBS



Proliferation was quantified as a measure of radioactive incorporation of ^{3}H-thymidine expressed in decays per minute (DPM). Values are mean ± SEM (n=5 for all treatments). 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2. All fatty acids were provided at a concentration of 60 μM, unless otherwise indicated. Treatments with different letters are significantly different ($p<0.05$).

D. Incorporation of CLA Mixtures into MDA-MB-231 Membrane Phospholipid

Supplementation with LA and the different CLA treatments resulted in elevated levels of these fatty acids in all membrane phospholipid fractions relative to the control treatment (Ctrl), which received only 5% FBS and no additional fatty acids (Tables 6-2, 6-3, 6-4 and 6-5). Even though LA and CLA was added in equal amounts into the culture, incorporation of LA was greater than total CLA levels in all fractions.

CLA treatments did not significantly reduce levels of LA relative to cells treated with LA(60) in all phospholipid fractions. All CLA treatments significantly reduced AA in PC. In PE and PS, only the Mix and Δ10*t*,12*c*-18:2 treatments reduced AA relative to cells treated with LA(60) or LA(120). There was no significant difference in levels of AA amongst treatments in PI.

Cells treated with Mix and Δ10*t*,12*c*-18:2 consistently showed significantly higher levels of LA relative to Δ9*c*,11*t*-18:2 treated cells in PC and PE, and the trend was also present in PI and PS. The corresponding increases in LA with Mix and Δ10*t*,12*c*-18:2 treatments were associated with a significant decrease of AA relative to Δ9*c*,11*t*-18:2 treatment in PE, and the trend persisted in PC, PI and PS.

Total n-6 fatty acids were only reduced in PC with CLA treatments (Figures 6-4, 6-5, 6-6 and 6-7). There was no consistent effect of CLA treatments on levels of total n-3 fatty acids. Surprisingly, the Mix treated cells incorporated significantly greater total CLA although the different CLA mixtures were supplemented at the same level (Figures 6-4, 6-5, 6-6 and 6-7). However, the magnitude difference was not great.

The presence of elongated, desaturated products from CLA were assessed by GC and GC-MS. Comparison of GC and GC-MS chromatographic traces between controls receiving only LA and cells treated with CLA did not indicate the presence of any new peaks. Analysis of prominent fragmentation ions by GC-MS also did not indicate the presence of CLA metabolites, predicted to be conjugated 18:3 and 20:4.

E. Time Course of CLA Incorporation into Membrane Phospholipid Fractions

The replication of each treatment at 5, 24 and 48 hrs was not sufficiently strong to demonstrate obvious trends in time and treatment effects on levels of LA, Δ9*c*,11*t*-18:2, Δ10*t*,12*c*-18:2, and AA (Table 6-6). Among these fatty acids of interest, there was only significant time effect due to Mix treatment on levels of AA in PC and CLA trans/trans in PI. There was no significant treatment effect at each time point, but there was a trend towards greater levels of LA in cells treated with Mix and Δ10*t*,12*c*-18:2 relative to cells treated with Δ9*c*,11*t*-18:2. There was also a trend showing that levels of AA tended to be lower in Mix and Δ10*t*,12*c*-18:2 treatments relative to cells treated with Δ9*c*,11*t*-18:2.

At 5 hrs, the incorporation of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 mimicked the ratio of these isomers in their original mixtures (Figure 6-8). At all time points, treatment with Mix, resulted in equal incorporation of both isomers, but the Δ9*c*,11*t*-18:2 isomer tended to be higher. Treatment with Δ9*c*,11*t*-18:2 increased levels of Δ9*c*,11*t*-18:2 and tended to be found in greater abundance relative to Δ10*t*,12*c*-18:2 but was not significantly different. At 5 hrs, treatment with Δ10*t*,12*c*-18:2, resulted in significantly greater incorporation of Δ10*t*,12*c*-18:2 relative to Δ9*c*,11*t*-18:2 in PC ($p<0.05$) and PI ($p<0.10$). This greater abundance was no longer apparent after 24 hours and levels of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 were not significantly different.

Table 6-2 Fatty Acid Composition of PC in MDA-MB-231 Cells

Fatty Acid	Ctrl	LA(60)	LA(120)	LA+Mix	LA+9c,11t	LA+10t,12c
C 16:0	22.4±0.7^{c}	22.8±0.8^{b}	25.3±1.6^{a}	23.0±0.8^{b}	23.7±1.1^{a}	23.0±0.5^{b}
C 17:0	0.32±0.04^{b}	0.50±0.03^{a}	0.49±0.02^{a}	0.49±0.03^{a}	0.52±0.02^{a}	0.52±0.01^{a}
C 18:0	12.8±0.7	15.1±0.5	14.1±1.0	15.2±0.6	14.6±0.5	15.2±0.4
C 24:0	0.30±0.02	0.37±0.04	0.42±0.13	0.31±0.05	0.23±0.04	0.37±0.04
C 16:1(7)	1.96±0.10^{a}	1.28±0.13^{b}	1.19±0.11^{c}	1.27±0.20^{bc}	1.48±0.10^{b}	1.26±0.12b^{c}
C 18:1(9)t	0^{c}	1.14±0.20^{ab}	0.86±0.15^{b}	1.34±0.29^{a}	1.00±0.10^{b}	1.26±0.19^{a}
C 18:1(9)	46.5±0.9^{a}	27.6±1.8^{c}	24.6±2.5^{d}	27.3±3.1^{c}	31.0±1.7^{b}	27.3±2.8^{c}
C 18:1(11)	2.68±0.95	3.64±0.09	3.50±0.09	3.50±0.13	3.41±0.11	3.54±0.08
C 20:1	0.60±0.09^{a}	0.86±0.63^{b}	0.79±0.64^{b}	0.92±0.76^{b}	0.99±0.81^{b}	0.87±0.69^{b}
C 22:1(9)	0.13±0.02	0.04±0.01	0.05±0.02	0.13±0.04	0.07±0.01	0.07±0.02
C 24:1(9)	0.09±0.03^{a}	0.05±0.02^{b}	0.04±0.02^{c}	0.05±0.02^{b}	0.05±0.02^{bc}	0.09±0.02^{ab}
C 18:2(6)	1.09±0.10^{c}	8.66±1.27^{b}	12.0±1.9^{a}	10.8±2.2^{a}	8.87±1.33^{b}	12.2±2.3^{a}
C 18:3(6)	0.46±0.07^{b}	1.00±0.25^{a}	0.74±0.02^{ab}	0.50±0.06^{b}	0.63±0.02^{b}	0.57±0.04^{b}
C 20:3(6)	0.86±0.05^{c}	1.79±0.44^{a}	1.74±0.44^{a}	0.86±0.20^{c}	1.28±0.32^{b}	0.95±0.22^{bc}
C 20:4(6)	2.35±0.13^{c}	5.42±0.31^{a}	5.41±0.37^{a}	2.84±0.14^{c}	3.68±0.24^{b}	3.15±0.13^{b}
C 22:2(6)	0.50±0.18	0.54±0.07	0.45±0.13	0.44±0.12	0.51±0.12	0.49±0.12
C 22:4(6)	0.47±0.15^{d}	1.66±0.40^{ab}	1.96±0.60^{a}	1.20±0.21^{b}	0.86±0.11^{cd}	1.18±0.23^{bc}
C 22:5(6)	1.36±0.64^{a}	1.08±0.27^{b}	1.00±0.20^{b}	0.84±0.18^{b}	0.84±0.10^{b}	0.83±0.18^{b}
C 18:3(3)	0.01±0.01	0.05±0.05	ND	0.01±0.01	ND	ND
C 20:5(3)	0.24±0.04	0.24±0.06	0.22±0.02	0.19±0.05	0.22±0.02	0.24±0.02
C 22:5(3)	0.69±0.08	1.15±0.05	1.00±0.13	0.91±0.05	0.73±0.10	0.94±0.02
C 22:6(3)	1.06±0.22	2.05±0.30	1.34±0.18	1.30±0.07	1.23±0.20	1.53±0.23
CLA Δ9*c*,11*t*	0.38±0.02^{b}	0.37±0.02^{b}	0.32±0.03^{b}	2.71±0.73^{a}	0.71±0.08^{b}	0.98±0.17^{b}
CLA Δ11*c*,13*t*	0.01±0.01	0.04±0.01	0.02±0.01	0.02±0.01	0.04±0.01	0.06±0.03
CLA Δ10*t*,12*c*	0.01±0.01^{c}	0.03±0.02^{c}	0^{c}	1.30±0.53^{a}	0.06±0.02^{c}	1.07±0.43^{b}
CLA cis/cis	0.12±0.04	0.04±0.02	0.02±0.01	0.12±0.05	0.35±0.08	0.02±0.01
CLA trans/trans	0.63±0.05^{a}	0.26±0.03^{b}	0.15±0.02^{cd}	0.21±0.08^{bc}	0.30±0.03^{b}	0.10±0.04^{d}

Values are mean ± SEM (n=4 for Ctrl and 5 for all other treatments). Within each row, treatments with different letters are significantly different ($p<0.05$). Ctrl – Control, no exogenous fatty acid ; 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2 ; ND - not detected. All fatty acids were provided at a concentration of 60 μM, unless otherwise indicated.

Table 6-3 Fatty Acid Composition of PE in MDA-MB-231 Cells

Fatty Acid	Ctrl	LA(60)	LA(120)	LA+Mix	LA+9c,11t	LA+10t,12c
C 16:0	3.73±0.59^{a}	3.77±0.10^{ac}	3.71±0.20^{c}	3.79±0.16^{b}	4.17±0.14^{ab}	4.25±0.24^{a}
C 17:0	0.28±0.05	0.49±0.02	0.45±0.06	0.48±0.04	0.44±0.04	0.45±0.03
C 18:0	14.2±1.2^{b}	21.3±0.1^{a}	20.6±0.2^{a}	20.6±0.6^{a}	19.9±1.0^{a}	20.3±1.0^{a}
C 16:1(7)	0.89±0.15^{a}	0.57±0.05^{ab}	0.49±0.04^{b}	0.45±0.06^{b}	0.49±0.04^{b}	0.50±0.08^{b}
C 18:1(9)t	0.29±0.11^{ab}	0.30±0.02^{ac}	0.24±0.03^{c}	0.41±0.05^{a}	0.31±0.03^{b}	0.38±0.04^{a}
C 18:1(9)	38.4±4.0^{a}	26.0±1.8^{b}	23.3±1.5^{c}	24.0±1.6^{bc}	25.5±1.2^{b}	22.7±2.6^{c}
C 18:1(7)	1.42±0.24b	1.40±0.03	1.44±0.10	1.53±0.11	1.38±0.10	1.48±0.07
C 20:1	0.08±0.04	ND	0.01±0.01	ND	ND	3.83±3.83
C 22:1(9)	0.52±0.12^{a}	0.44±0.07^{a}	0.43±0.03^{a}	0.47±0.05^{a}	0.41±0.07^{ab}	0.35±0.04^{c}
C 24:1(9)	0.05±0.05	ND	ND	ND	ND	ND
C 18:2(6)	0.60±0.06^{c}	5.46±0.80^{b}	7.29±1.20^{a}	7.23±1.41^{a}	5.96±1.02^{b}	7.87±1.13^{a}
C 18:3(6)	0.29±0.15	0.36±0.01	0.51±0.16	0.39±0.11	0.36±0.02	0.34±0.03
C 20:3(6)	2.13±1.16	2.33±0.08	2.35±0.08	1.59±0.10	2.60±0.24	1.68±0.14
C 20:4(6)	21.9±5.8^{a}	18.5±0.8^{b}	19.5±0.6^{b}	14.9±0.6^{c}	19.3±1.8^{b}	15.0±0.9^{c}
C 22:2(6)	1.04±0.15	0.91±0.11	0.89±0.05	0.78±0.05	1.22±0.32	0.75±0.10
C 22:4(6)	2.40±0.21^{c}	5.11±0.79^{b}	6.73±0.54^{a}	5.26±0.54^{b}	4.98±0.75^{b}	5.26±0.56^{b}
C 22:5(6)	1.28±0.40^{a}	0.60±0.14^{b}	0.61±0.11^{b}	0.55±0.11^{b}	0.60±0.16^{b}	0.48±0.10^{b}
C 18:3(3)	0.04±0.01	ND	0.02±0.01	ND	0.01±0.01	0.12±0.09
C 20:5(3)	0.93±0.14^{a}	0.84±0.12^{b}	0.59±0.11^{c}	0.72±0.10^{bc}	0.90±0.15^{ab}	0.70±0.11^{c}
C 22:5(3)	2.88±0.17	3.21±0.12	3.06±0.11	4.01±0.13	3.06±0.59	4.32±0.28
C 22:6(3)	4.87±0.51^{b}	5.55±0.08^{b}	5.18±0.20^{b}	7.29±0.79^{a}	5.58±0.46^{b}	6.08±0.41^{b}
CLA Δ9*c*,11*t*	0.07±0.02^{b}	0.08±0.02^{b}	0.09±0.01^{b}	2.20±0.68^{a}	0.49±0.12^{b}	0.63±0.13^{b}
CLA Δ11*c*,13*t*	0.09±0.01	0.07±0.02	0.11±0.02	0.07±0.02	0.09±0.01	0.16±0.10
CLA Δ10*t*,12*c*	0.02±0.01^{b}	0^{b}	0.05±0.03^{b}	1.32±0.59^{a}	0.07±0.02^{b}	0.96±0.38^{a}
CLA cis/cis	0.08±0.03^{bc}	0.02±0.02^{c}	0.02±0.01^{c}	0.15±0.04^{ab}	0.22±0.05^{a}	0.04±0.01^{c}
CLA trans/trans	0.06±0.03	0.23±0.04	0.14±0.07	0.23±0.04	0.29±0.06	0.13±0.11

Values are mean ± SEM (n=4 for Ctrl and 5 for all other treatments). Within each row, treatments with different letters are significantly different ($p<0.05$). Ctrl – Control, no exogenous fatty acid ; 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2 ; ND - not detected. All fatty acids were provided at a concentration of 60 μM, unless otherwise indicated.

Table 6-4 Fatty Acid Composition of PI in MDA-MB-231 Cells

Fatty Acid	Ctrl	LA(60)	LA(120)	LA+Mix	LA+9c,11t	LA+10t,12c
C 16:0	8.55±0.46	5.26±0.26	7.79±3.35	6.05±0.48	6.08±0.94	5.47±0.70
C 17:0	0.24±0.02	0.46±0.07	0.38±0.04	0.45±0.04	0.45±0.03	0.45±0.04
C 18:0	25.9±1.7	27.7±1.3	25.8±2.8	29.9±0.7	28.7±1.0	29.2±1.6
C 24:0	0.61±0.21	0.95±0.42	0.44±0.08	0.57±0.13	0.57±0.20	1.05±0.55
C 16:1(7)	0.41±0.06	0.31±0.08	0.25±0.08	0.45±0.12	0.37±0.07	0.18±0.06
C 18:1(9)t	0.87±0.09^{a}	0.70±0.10^{b}	0.36±0.11^{b}	0.64±0.08^{a}	0.53±0.06^{ab}	0.72±0.08^{b}
C 18:1(9)	29.5±1.8	18.2±3.5	20.3±4.0	16.9±2.7	17.0±5.0	16.3±3.1
C 18:1(7)	2.35±0.26	1.76±0.14	1.38±0.35	1.84±0.10	4.54±2.73	1.74±0.17
C 20:1	0.95±0.08^{a}	0.22±0.07^{bc}	0.18±0.07^{c}	0.44±0.06^{b}	0.10±0.08^{cd}	0.02±0.02^{d}
C 22:1(9)	0.65±0.12	0.26±0.14	0.38±0.11	0.65±0.31	0.55±0.12	0.43±0.15
C 24:1(9)	0.07±0.02^{a}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}
C 18:2(6)t/t	ND	0.16±0.02	0.18±0.06	0.30±0.15	0.69±0.51	0.19±0.06
C 18:2(6)	0.35±0.06^{d}	2.35±0.56^{c}	4.25±1.32^{a}	3.81±1.23^{ab}	2.78±0.61^{bc}	3.88±1.14^{a}
C 18:3(6)	0.18±0.01	0.26±0.01	0.27±0.03	0.43±0.09	0.33±0.06	0.16±0.04
C 20:3(6)	1.70±0.15	4.15±0.50	4.52±0.76	3.20±0.22	3.71±0.98	3.47±0.65
C 20:4(6)	5.95±0.94^{b}	12.32±2.00^{a}	14.13±2.74^{a}	10.77±1.03^{a}	12.17±1.30^{a}	10.98±1.37^{a}
C 22:2(6)	1.61±0.42	1.93±0.27	1.96±0.72	1.66±0.14	1.49±0.12	1.71±0.03
C 22:4(6)	2.41±1.18	3.81±1.28	2.57±0.38	2.28±0.30	3.23±0.80	4.07±1.01
C 22:5(6)	0.76±0.09	5.81±3.36	2.17±0.84	2.77±1.01	2.76±1.07	4.44±2.12
C 18:3(3)	ND	0.08±0.03	ND	0.13±0.08	0.03±0.03	0.04±0.03
C 20:5(3)	ND	0.14±0.04	0.18±0.04	0.15±0.05	0.35±0.09	0.22±0.07
C 22:5(3)	1.11±0.21	0.94±0.12	0.80±0.14	1.06±0.11	1.08±0.06	1.01±0.15
C 22:6(3)	0.80±0.17	1.06±0.15	0.86±0.09	1.26±0.35	1.08±0.20	1.63±0.35
CLA Δ9*c*,11*t*	0.03±0.03^{a}	0.03±0.01^{b}	0.03±0.02^{b}	0.68±0.20^{a}	0.15±0.02^{b}	0.21±0.06^{b}
CLA Δ11*c*,13*t*	0.01±0.01	0.12±0.04	0.11±0.03	0.12±0.05	0.12±0.03	0.17±0.02
CLA Δ10*t*,12*c*	0.09±0.03^{b}	0.04±0.02^{b}	0.01±0.01^{b}	0.57±0.26^{a}	0.06±0.03^{b}	0.49±0.19^{a}
CLA cis/cis	ND	0.04±0.02	0.07±0.05	0.23±0.01	0.24±0.04	0.28±0.18
CLA trans/trans	1.74±0.15^{a}	0.49±0.15^{b}	0.31±0.11^{b}	0.84±0.25^{b}	0.55±0.18^{b}	0.31±0.08^{b}

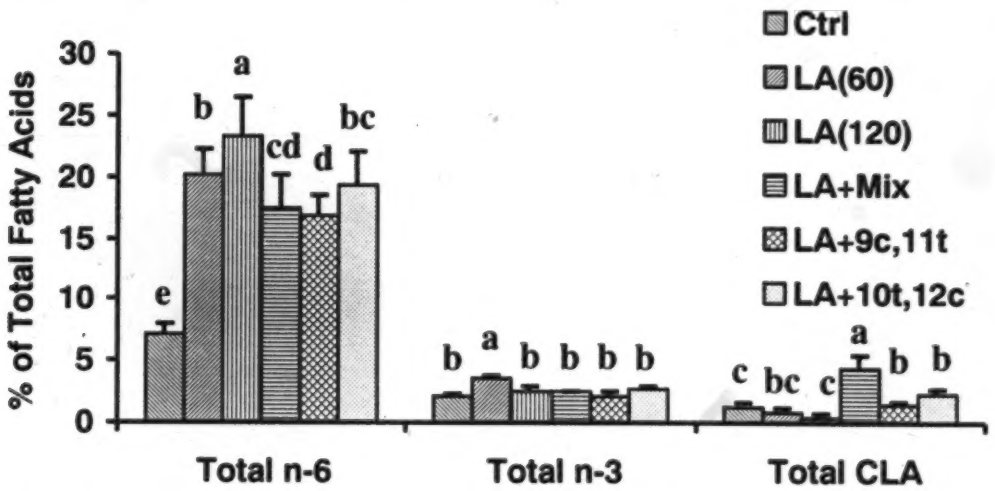
Values are mean ± SEM (n=4 for Ctrl and 5 for all other treatments). Within each row, treatments with different letters are significantly different ($p<0.05$). Ctrl – Control, no exogenous fatty acid ; 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2 ; ND - not detected. All fatty acids were provided at a concentration of 60 μM, unless otherwise indicated.

Table 6-5 Fatty Acid Composition of PS in MDA-MB-231 Cells

Fatty Acid	Ctrl	LA(60)	LA(120)	LA+Mix	LA+9c,11t	LA+10t,12c
C 16:0	3.49±0.68^{c}	3.23±0.30^{bc}	4.86±0.25^{a}	4.30±0.50^{ab}	3.65±0.14^{b}	4.42± 0.51^{a}
C 17:0	0.37±0.04^{c}	0.60±0.03^{a}	0.53±0.02^{ab}	0.50±0.04^{b}	0.48±0.03^{b}	0.51± 0.06^{b}
C 18:0	31.7±0.7^{b}	34.6±2.1^{a}	34.7±1.5^{a}	36.4±1.6^{a}	33.5±1.8^{a}	33.5± 0.8^{ab}
C 16:1(7)	0.85±0.21	0.31±0.09	0.50±0.21	0.63±0.27	0.50±0.09	1.03± 0.67
C 18:1(9) trans	0.38±0.08	0.41±0.18	0.31±0.15	0.39±0.20	0.36±0.15	0.47± 0.22
C 18:1(9)	43.1±0.8^{a}	24.9±1.1^{b}	24.2±2.6^{b}	24.4±3.3^{b}	27.3±1.9^{b}	27.9± 0.9^{b}
C 18:1(7)	1.39±0.33	1.22±0.07	1.19±0.07	1.29±0.05	1.17±0.12	1.32± 0.06
C 20:1	0.09±0.03	0.08±0.02	0.16±0.03	0.08±0.03	0.10±0.03	0.05± 0.03
C 22:1(9)	1.86±0.31	1.85±0.43	1.24±0.19	1.43±0.20	1.68±0.41	1.36± 0.22
C 24:1(9)	0.39±0.13^{a}	0.11±0.01^{b}	0.18±0.02^{ab}	0.28±0.08^{a}	0.24±0.05^{a}	0.09± 0.01^{b}
C 18:2(6)	0.59±0.07^{c}	5.46±0.64^{ab}	6.05±1.10^{a}	5.24±1.13^{b}	4.81±0.80^{b}	6.74± 1.26^{a}
C 18:3(6)	1.15±0.22	0.83±0.13	1.13±0.40	0.90±0.06	0.67±0.10	0.69± 0.20
C 20:3(6)	1.49±0.44^{b}	2.87±0.63^{a}	4.27±1.07^{a}	2.44±0.51^{b}	2.82±0.33^{ab}	2.00± 0.20^{b}
C 20:4(6)	1.27±0.28^{c}	3.71±0.31^{a}	3.78±0.54^{a}	2.22±0.10^{bc}	2.83±0.55^{ab}	2.35± 0.18^{b}
C 22:2(6)	0.02±0.01	0.02±0.02	ND	ND	ND	ND
C 22:4(6)	2.74±1.74	4.59±0.50	6.17±1.72	5.21±1.31	6.15±0.95	4.97± 1.50
C 22:5(6)	0.34±0.03^{b}	0.51±0.08^{a}	0.60±0.12^{a}	0.53±0.07^{a}	0.59±0.04^{a}	0.57± 0.03^{a}
C 22:5(3)	0.72±0.20	1.75±0.06	1.84±0.45	1.60±0.05	2.12±0.60	1.74± 0.14
C 22:6(3)	2.96±0.89	6.85±2.58	3.38±0.70	3.67±0.50	5.32±1.25	2.85± 0.07
CLA Δ9*c*,11*t*	0.07±0.02^{b}	0.07±0.01^{b}	0.06±0.003^{b}	1.64±0.45^{a}	0.32±0.10^{b}	0.33± 0.08^{b}
CLA Δ11*c*,13*t*	0.21±0.04	0.21±0.03	0.26±0.05	0.21±0.02	0.19±0.05	0.25± 0.05
CLA Δ10*t*,12*c*	0.01±0.01^{b}	0.04±0.02^{b}	0.06±0.03^{b}	0.84±0.36^{a}	0.08±0.01^{b}	0.78± 0.33^{a}
CLA cis/cis	0.02±0.02	0.10±0.08	0.04±0.04	0.08±0.03	0.16±0.05	0.03± 0.01
CLA trans/trans	0.21±0.17	0.22±0.07	0.18±0.05	0.15±0.04	0.19±0.10	0.10± 0.03

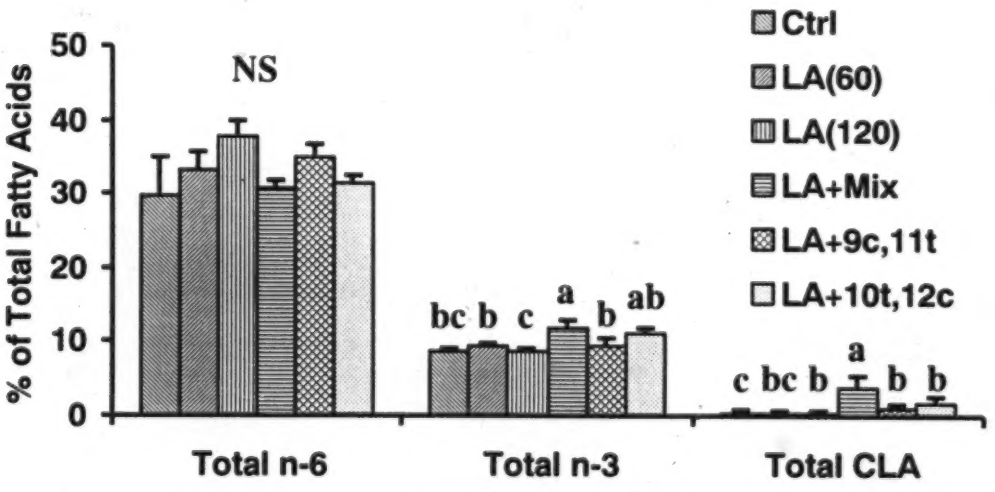
Values are mean ± SEM (n=4 for Ctrl and 5 for all other treatments). Within each row, treatments with different letters are significantly different ($p<0.05$). Ctrl – Control, no exogenous fatty acid ; 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2 ; ND - not detected. All fatty acids were provided at a concentration of 60 μM, unless otherwise indicated.

Figure 6-4 Total Content of n-6, n-3 and CLA Fatty Acids in PC



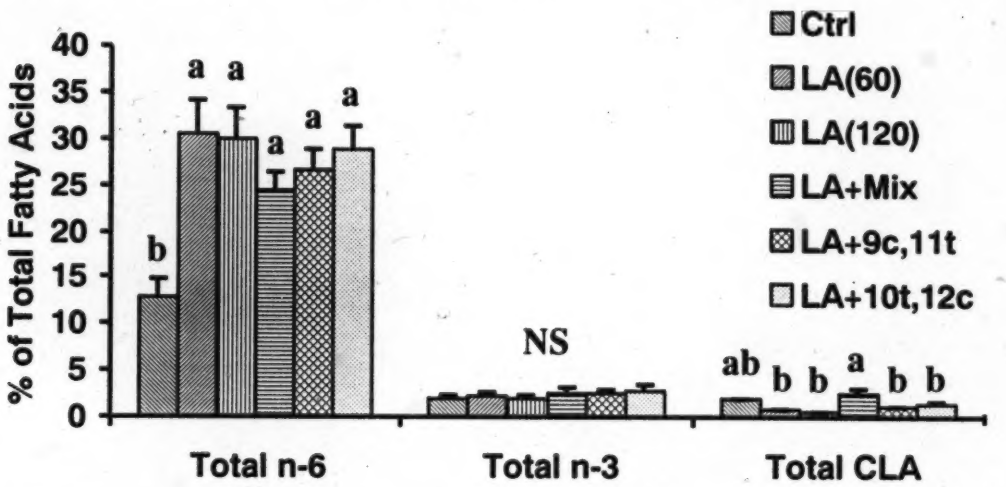
Values are mean ± SEM (n=4 for Ctrl and 5 for all other treatments). Within each group, different letters are significantly different ($p<0.05$). Ctrl – Control, no exogenous fatty acid ; 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2.

Figure 6-5 Total Content of n-6, n-3 and CLA Fatty Acids in PE



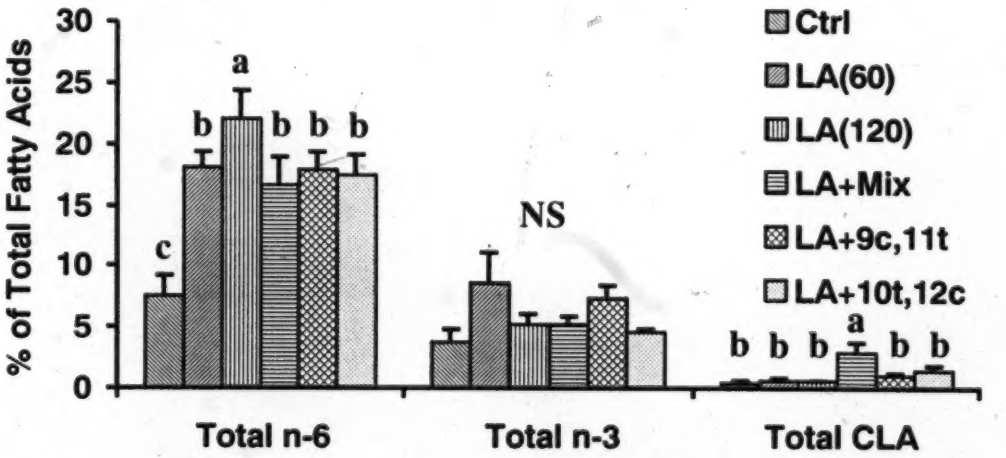
Values are mean ± SEM (n=4 for Ctrl and 5 for all other treatments). Within each group, different letters are significantly different ($p<0.05$). Ctrl – Control, no exogenous fatty acid ; 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2.

Figure 6-6 Total Content of n-6, n-3 and CLA Fatty Acids in PI



Values are mean ± SEM (n=4 for Ctrl and 5 for all other treatments). Within each group, different letters are significantly different ($p<0.05$). Ctrl – Control, no exogenous fatty acid ; 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mixture – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2.

Figure 6-7 Total Content of n-6, n-3 and CLA Fatty Acids in PS



Values are mean ± SEM (n=4 for Ctrl and 5 for all other treatments). Within each group, different letters are significantly different ($p<0.05$). Ctrl – Control, no exogenous fatty acid ; 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mixture – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2.

Table 6-6 Levels of LA, AA and CLA in MDA-MB-231 Membrane Phospholipid Fractions Over 48hrs

	LA+Mix			LA+9c,11t			LA+10t,12c		
PC	5hrs	24hrs	48hrs	5hrs	24hrs	48hrs	5hrs	24hrs	48hrs
C 18:2(6)	13.1±0.2	17.6±5.6	12.1±2.8	13.3±1.0	14.8±4.3	9.9±2.8	15.1±1.2	20.7±4.6	11.4±1.8
C 20:4(6)	4.90±0.22^a	3.09±0.68^b	2.70±0.05^b	5.29±0.72	4.47±0.35	3.45±0.46	4.68±0.60	3.83±1.02	3.46±0.45
CLA Δ9*c*,11*t*	2.75±0.82	5.61±1.92	2.58±0.85	1.63±0.55	0.70±0.40	1.99±1.40	1.20±0.10	1.00±0.61	0.78±0.41
CLA Δ11*c*,13*t*	ND	0.12±0.12	0.13±0.13	0.05±0.05	0.18±0.11	0.03±0.03	ND	0.16±0.16	0.59±0.41
CLA Δ10*t*,12*c*	2.12±1.05	4.06±2.21	1.94±0.65	0.69±0.57	0.07±0.05	1.06±0.78	2.95±0.52	1.79±1.28	0.83±0.45
CLA cis/cis	0.94±0.80	0.09±0.05	0.22±0.12	0.28±0.18	0.36±0.19	0.18±0.09	ND	0.03±0.03	0.19±0.19
CLA trans/trans	0.08±0.05	0.29±0.11	0.11±0.02	0.09±0.05	0.09±0.05	0.44±0.34	0.66±0.34	0.25±0.18	0.08±0.01
PE									
C 18:2(6)	2.83±0.55^b	8.43±1.76^a	8.62±1.88^a	2.77±0.35^b	6.59±0.90^a	7.10±1.96^a	3.24±0.53^b	7.07±0.57^a	8.23±1.22^a
C 20:4(6)	17.7±0.4	13.9±2.1	14.7±0.4	15.8±3.6	17.1±1.2	17.0±1.8	18.0±1.0	15.5±0.6	16.3±0.4
CLA Δ9*c*,11*t*	0.33±0.33	3.12±1.06	2.10±0.71	0.12±0.12	0.25±0.14	1.45±1.12	0^b	0.56±0.15^a	0.69±0.14^a
CLA Δ11*c*,13*t*	ND	ND	ND	ND	ND	ND	ND	0.17±0.17	ND
CLA Δ10*t*,12*c*	0.34±0.34	3.35±2.10	1.79±0.62	ND	ND	0.89±0.89	0.44±0.44	1.67±1.00	0.72±0.40
CLA cis/cis	ND	ND	ND	ND	ND	ND	ND	0.20±0.20	0.06±0.06
CLA trans/trans	ND	ND	ND	ND	ND	ND	ND	ND	ND
PI									
C 18:2(6)	9.79±1.99	9.24±3.17	6.52±1.45	6.85±1.41	6.17±1.54	4.46±1.40	6.58±2.34	9.49±2.50	5.88±0.67
C 20:4(6)	20.3±3.0	13.4±2.0	14.6±0.6	16.2±1.7	19.1±2.9	15.2±1.1	14.3±5.1	18.4±0.45	17.6±1.2
CLA Δ9*c*,11*t*	1.20±0.51	1.33±0.88	0.74±0.43	0.48±0.36	0.25±0.25	0.40±0.40	0.22±0.11	0.18±0.18	ND
CLA Δ11*c*,13*t*	ND	ND	0.04±0.04	ND	ND	ND	ND	ND	ND
CLA Δ10*t*,12*c*	2.71±1.35	1.42±1.11	0.89±0.45	0.61±0.53	ND	0.41±0.41	2.01±0.84	1.21±0.57	0.26±0.26
CLA cis/cis	0.26±0.14	ND	0.02±0.02	0.13±0.07	0.03±0.03	ND	0.04±0.04	ND	ND
CLA trans/trans	1.09±0.37^a	0.08±0.08^b	0.07±0.07^b	0.25±0.20	ND	ND	0.23±0.23	0.12±0.12	ND
PS									
C 18:2(6)	3.03±0.03	8.84±1.90	6.45±1.41	1.46±0.80	5.45±0.95	3.94±1.52	3.17±0.08	7.26±1.58	5.88±1.07
C 20:4(6)	2.14±0.29	1.67±0.23	1.62±0.05	1.83±0.13	2.02±0.34	1.72±0.28	2.57±0.37	1.73±0.19	1.89±0.19
CLA Δ9*c*,11*t*	0.32±0.23	2.70±1.06	1.67±0.45	ND	0.09±0.07	0.83±0.83	0.23±0.12	0.25±0.25	ND
CLA Δ11*c*,13*t*	0.03±0.03	ND	ND	ND	0.11±0.07	ND	0.01±0.01	ND	ND
CLA Δ10*t*,12*c*	0.33±0.29	2.66±1.53	1.50±0.43	ND	ND	0.51±0.51	0.24±0.24	0.96±0.75	0.33±0.33
CLA cis/cis	0.27±0.24	0.18±0.18	ND	ND	0.17±0.17	ND	0.58±0.39	ND	ND
CLA trans/trans	ND	ND	ND	ND	0.03±0.03	ND	0.15±0.08	ND	ND

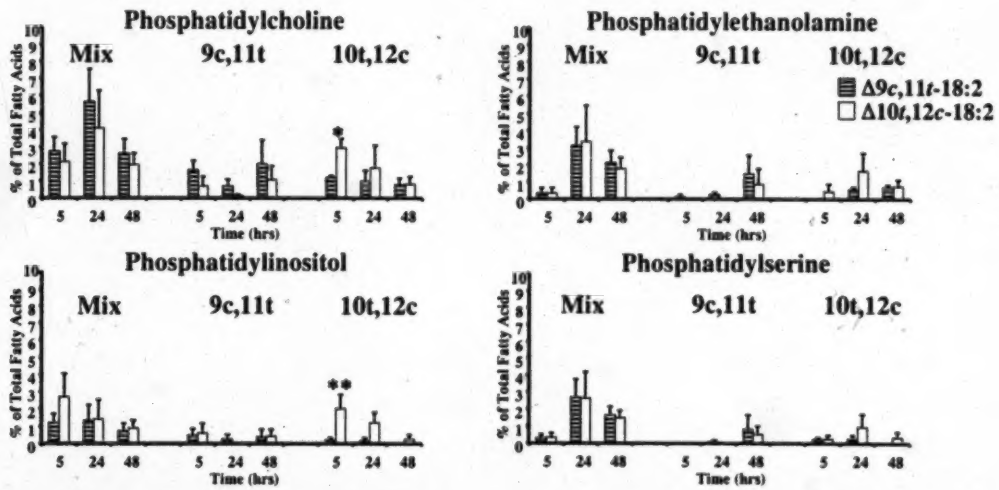
Values are mean ± SEM (n=3 for each time point for each treatment). Within each row of each treatment, treatments having different letters are significantly different ($p<0.05$). 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2 ; ND - not detected. All fatty acids were provided at a concentration of 60 μM, unless otherwise indicated.

Table 6-7 Index of Formation of AA from LA

Treat	PC	PE	PI	PS
Ctrl	2.20±0.15[a]	38.88±12.13[a]	18.47±4.61[a]	2.17±0.42[a]
LA(60)	0.68±0.11[b]	3.61±0.41[b]	5.76±0.60[b]	0.70±0.06[b]
LA(120)	0.48±0.06[c]	2.96±0.43[b]	4.32±0.91[bc]	0.73±0.22[b]
LA+Mix	0.32±0.08[d]	2.48±0.61[b]	3.95±0.87[c]	0.54±0.14[b]
LA+9c,11t	0.46±0.09[c]	3.99±1.26[b]	4.94±0.69[b]	0.60±0.08[b]
LA+10t,12c	0.30±0.06[d]	2.13±0.41[b]	3.52±0.72[c]	0.39±0.06[b]

Values are mean ± SEM (n=4 for Ctrl and 5 for all other treatments). Within each column, treatments with different letters are significantly different ($p<0.05$). Ctrl – Control, no exogenous fatty acid ; 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2. All fatty acids were provided at a concentration of 60 μM, unless otherwise indicated. The ratio of AA to LA expressed as the ratio of % AA to % LA of total fatty acids is an index of conversion of LA to AA by elongation and desaturation.

Figure 6-8 Incorporation of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 over Time into Phospholipid Fractions of MDA-MB-231 Treated with Different Mixtures of Isomers



Values are Mean ± SEM. (n=3 for each time point for each treatment) 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2. All fatty acids were provided at 60 μM. There was no significant effect of treatment or time by 2-way ANOVA, blocked by passage. Levels of Δ9*c*,11*t*-18:2, and Δ10*t*,12*c*-18:2 were compared by 1-way ANOVA, blocked by passage at each time point, *$p<0.05$, **$p<0.10$.

Table 6-8 Index of Formation of AA from LA Over Time

PC

Time (hrs)	LA+Mix	LA+9c,11t	LA+10t,12c
5	0.37 ± 0.02	0.40 ± 0.06	0.32 ± 0.05
24	0.23 ± 0.08[b]	0.36 ± 0.10[a]	0.20 ± 0.04[b]
48	0.26 ± 0.08	0.42 ± 0.14	0.31 ± 0.04

PE

Time (hrs)	LA+Mix	LA+9c,11t	LA+10t,12c
5	6.92 ± 1.70	5.78 ± 1.44	5.92 ± 1.14
24	1.87 ± 0.54	2.74 ± 0.54	2.21 ± 0.15
48	1.97 ± 0.61	2.87 ± 0.86	2.08 ± 0.34

PI

Time (hrs)	LA+Mix	LA+9c,11t	LA+10t,12c
5	2.11 ± 0.10	2.51 ± 0.43	2.17 ± 0.14
24	1.98 ± 0.92[b]	3.66 ± 1.12[a]	2.18 ± 0.46[a]
48	2.51 ± 0.61	4.16 ± 1.20	3.04 ± 0.31

PS

Time (hrs)	LA+Mix	LA+9c,11t	LA+10t,12c
5	0.71 ± 0.10	0.93 ± 0.34	0.81 ± 0.11
24	0.21 ± 0.05	0.37 ± 0.04	0.27 ± 0.08
48	0.28 ± 0.07	0.70 ± 0.37	0.34 ± 0.06

The metabolism of LA to AA via elongation and desaturation is indirectly assessed as the ratio of AA to LA. MDA-MB-231 cells were treated with one of three different CLA mixtures and determinations were made at 5, 24 and 48 hours for membrane phospholipid fractions of PC, PE, PI and PS. Values are mean ± SEM (n=3 for each treatment at each time). Within a row, treatments with different letters are significantly different ($p<0.05$). Ctrl – Control, no exogenous fatty acid ; 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2. All fatty acids were provided at a concentration of 60 μM, unless otherwise indicated. The ratio of AA to LA expressed as the ratio of % AA to % LA of total fatty acids is an index of conversion of LA to AA by elongation and desaturation.

F. Effect of CLA Mixtures on PGE_2 Synthesis by MDA-MB-231 Cells

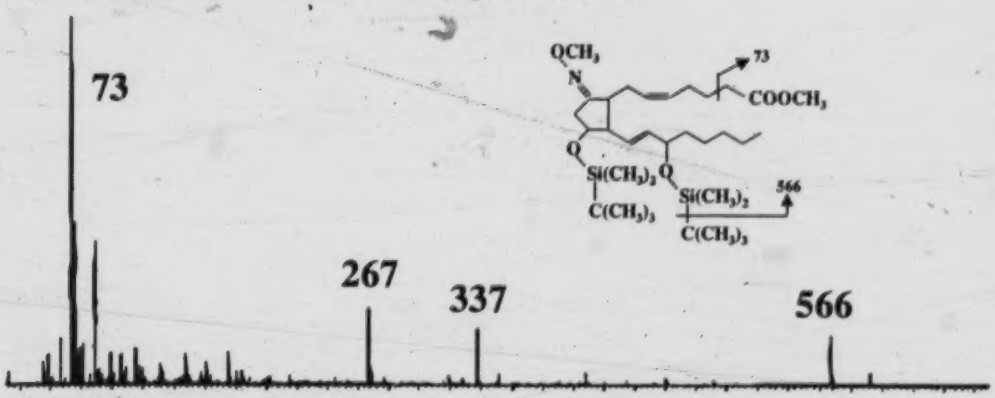
The rationale for using both a calcium ionophore and phorbol ester was to ensure that PGE_2 synthesis was stimulated so that sufficient amounts could be detected. It can be noted in a separate experiment that the calcium ionophore alone was not sufficiently potent to stimulate PGE_2 synthesis (Appendix H). Activation of PGE_2 synthesis results in intracellular calcium release which can be stimulated by the calcium ionophore, but it appears that PKC and PLC [34] activation by phorbol esters play a larger role in stimulating PGE_2 synthesis in this cell line. The inclusion of exogenous AA was both a control to show that the MDA-MB-231 cells could produce PGE_2 and to ensure that an adequate amount of product could be extracted and detected for later analysis. The level of AA used was shown not to have any adverse effects during the 24 hour incubation used in this study (Appendix F).

The only eicosanoid found in the culture media of stimulated MDA-MB-231 cells was PGE_2. PGE_2 was readily identified by its characteristic ions by GC-MS as well as 11-deoxy-PGE_1, the internal standard (Table 6-1). An ion scan of derivatized PGE_2 is shown (Figure 6-9). Cells treated with AA alone were used as a baseline measure demonstrating that the MDA-MB-231 cells were capable of synthesizing PGE_2. The addition of LA at 60 or 120 μM significantly ($p<0.05$) increased PGE_2 synthesis relative to AA alone (Figure 6-10). All CLA treatments significantly ($p<0.05$) inhibited the synthesis of PGE_2 relative to AA+LA(60) and AA+LA(120) treatments, but were not different from AA alone. GC-MS analysis did not indicate that CLA was converted into new prostaglandins or leukotrienes.

In early pilot work using enzyme immunoassays as a means to detect these changes in PGE_2 and LTB_4 (Appendix G), similar type responses were observed for the inhibitory effect of CLA on PGE_2 and absence of effect on LTB_4. Reproduction of these effects on the final treatments of interest utilizing co-treatment of CLA with LA had an opposite effect and actually significantly increased both PGE_2 and LTB_4 synthesis. These observations were difficult to resolve and may have been attributed to variability of the antibodies from different lots, which lead to the decision to use GC-MS in the present study. GC-MS provides both retention time and structural information to identify the

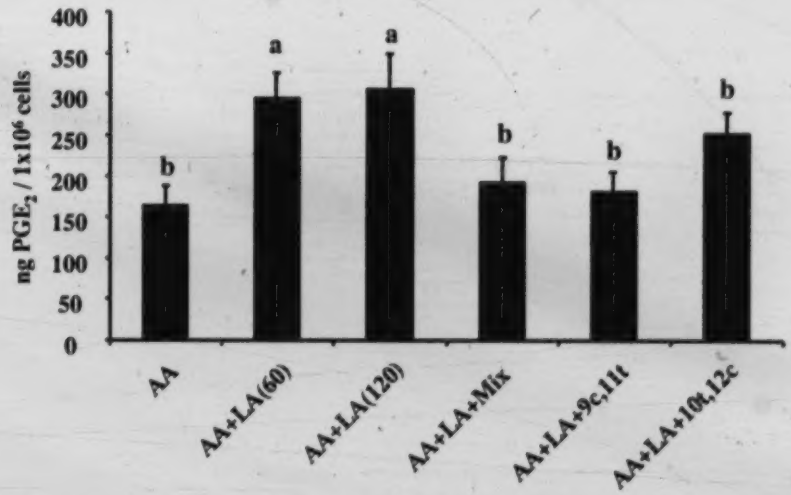
eicosanoids of interest whereas antibody detection techniques may have unanticipated cross reactivity with compounds not previously tested.

Figure 6-9 GC-MS Total Ion Chromatogram of PGE_2



The GC-MS fragmentation pattern of PGE_2 and its derivatized structure is shown above.

Figure 6-10 Effect of CLA Mixtures on PGE_2 Production by MDA-MB-231 Cells



Values are Mean ± SEM (n=6-7 for each treatment). Different letters are significantly different ($p<0.05$). 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2. All fatty acids were provided at a concentration of 60 μM, unless otherwise indicated.

5. Discussion

The main objective of the present study was to gain a greater understanding of the mechanism of CLA action by examining how the specific isomers, Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 affect availability of LA and AA in membrane phospholipid fractions, consequently altering PGE_2 synthesis and tumor growth.

To examine the competitive incorporation of LA and CLA, it was necessary to provide both fatty acids equally in culture. Previous work by Winchell et al. [35] showed that LA supplemented to MDA-MB-231 cells from a range of 60-120 μM was not cytotoxic. CLA at 60 μM in culture is within reported physiological levels [36-38] but LA at this concentration is not physiological and is typically found in serum in the mM range [39]. The isomeric similarities shared by LA and CLA would suggest that they both might compete for uptake into membrane phospholipid. The present study clearly demonstrates that on an equal molar basis LA was preferentially incorporated into all phospholipid fractions relative to CLA (Tables 6-2, 6-3, 6-4 and 6-5). Therefore, CLA does not simply displace LA, thereby reducing substrate for the synthesis of AA as a mechanism of action.

Addition of LA, 60 or 120 μM, increased LA in PC, PE, and PI, which increased the level of AA (Tables 6-2, 6-3, 6-4 and 6-5). There was no evidence to show that CLA treatments decreased LA, but there was a strong indication showing that CLA treatments decreased AA content in membrane phospholipid fractions. The main finding of the present study is that the mechanism of action was not the reduction in the availability of LA. The mechanism of action may have been the reduction of AA though inhibition of LA to AA metabolism, and appears to be a specific effect of the Δ10*t*,12*c*-18:2 isomer. Both the Mix and Δ10*t*,12*c*-18:2 treatments consistently increased the phospholipid content of LA and decreased levels of AA in membrane phospholipid fractions relative to Δ9*c*,11*t*-18:2 treated cells. The enhanced levels of LA caused by the Mix and Δ10*t*,12*c*-18:2 treatments suggest that elongation and desaturation of LA was inhibited, resulting in an accumulation of LA and reduction of AA. To obtain an indirect measure of elongation and desaturation, metabolism of LA to AA was evaluated as a ratio of the product AA to the substrate, LA (Table 6-7) [40]. Metabolism of LA to AA was significantly reduced by

the Mix and Δ10*t*,12*c*-18:2 treatments in the PC fraction. Although not significant, similar trends were also found in PE, PI and PS. Metabolism of LA to AA in the Δ9*c*,11*t*-18:2 treatments were similar to LA(60) and LA(120) treatments, showing that the Δ9*c*,11*t*-18:2 isomer did not inhibit LA to AA conversion (Table 6-7). This is consistent with the observation that levels of LA and AA in Δ9*c*,11*t*-18:2 treated cells tended to be similar to those in LA(60) and LA(120) treated cells. There was only one instance where AA was reduced by Δ9*c*,11*t*-18:2. In the PC fraction, Δ9*c*,11*t*-18:2 reduced AA relative to LA(60) and LA(120) treatments (Table 6-2). This may be due to an effect of some Δ10*t*,12*c*-18:2 which was also present in a small amount in the mixture. Cumulatively, these observations suggest that the Δ10*t*,12*c*-18:2 isomer, which was present in both the Mix and Δ10*t*,12*c*-18:2 treatments, was responsible for the reduction in AA.

The initial assessment of membrane incorporation of CLA was determined at 48 hrs. To determine if the effects of the Δ10*t*,12*c*-18:2 was transient, a time course experiment was conducted. Although not significant, at 5, 24 and 48 hrs, similar trends towards higher levels of LA and lower levels of AA were observed with the Mix and Δ10*t*,12*c*-18:2 treatments relative to the Δ9*c*,11*t*-18:2 treatment (Table 6-6) (see Appendix J for all fatty acids). When these values were re-expressed as the ratio of AA to LA, as an indirect measure of desaturation and elongation of LA to AA (Table 6-8), the Mix and Δ10*t*,12*c*-18:2 treatments reduced LA to AA metabolism relative to Δ9*c*,11*t*-18:2 treatment in PC at 24 hrs. Although not significant, this general trend was consistently observed at all other time points and fractions.

The results of this study confirms and extends upon the main finding made by Banni et al. [6] showing that CLA does not displace LA but inhibits the elongation and desaturated cascade converting LA to AA. Banni et al. [6] showed that CLA reduced 18:3(n6), 20:3(n6) and AA in mammary tissue when rats were fed a mixture of CLA isomers. The authors noted that they could not discern specific effects related to the main isomers present within the mixture. The present study extends the findings made by Banni et al [6] by providing evidence which suggests that the Δ10*t*,12*c*-18:2 isomer is the likely isomer responsible for affecting essential fatty acid metabolism. Coinciding with increasing levels of CLA supplementation, increasing amounts of conjugated 18:3 and

20:3 derived from CLA were also detected by Banni et al. [6]. The authors noted that only a small fraction of CLA was converted to elongated/desaturated products. GC-MS analysis in the present study did not reveal the presence of any conjugated products derived from CLA. This may have been due to limitations in detection from cells obtained from cell culture versus the analysis of whole tissues by Banni et al. [6].

The time course analysis of the incorporation of the Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 showed rapid changes over time favoring the retention of Δ9*c*,11*t*-18:2. Initially at 5 hrs, the membrane composition reflected the composition of the CLA mixture supplemented into the media of high, low or equal amounts of Δ9c,11*t*- and Δ10*t*,12*c*-18:2. With Δ10*t*,12*c*-18:2 treatment, the level of Δ10*t*,12*c*-18:2 was significantly greater than Δ9*c*,11*t*-18:2 in PC at 5 hrs and this difference was no longer apparent by 24 hrs (Figure 6-8). This indicates that Δ10*t*,12*c*-18:2 is initially incorporated into membrane phospholipid but is rapidly turned over, whereas Δ9*c*,11*t*-18:2 is retained longer. This finding confirms other studies showing preferential incorporation of Δ9*c*,11*t*-18:2 into membrane phospholipid [3; 4; 21]. In a recent study by De Deckere et al. [41] feeding an equal mixture of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 or mixtures enriched in either Δ9*c*,11*t*-18:2 or Δ10*t*,12*c*-18:2 to hamsters, it was reported that Δ9*c*,11*t*-18:2 was preferentially incorporated into membrane phospholipid. It was interesting to note that the level of enrichment of Δ10*t*,12*c*-18:2 isomer was 8 fold greater than Δ9*c*,11*t*-18:2, yet the level of incorporation was only enhanced approximately 2 fold in total liver phospholipid and triglyceride and lipoprotein fractions. The authors suggest that the lower incorporation of Δ10*t*,12*c*-18:2 was due to increased oxidation *in vivo* [41].

The proliferation experiments showed that both Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2 treatments significantly inhibited the growth of MDA-MB-231 cells relative to LA(60) and LA(120) treatments (Figure 6-3). Although both of these CLA treatments inhibited cell growth similarly, the magnitude of inhibition was slightly greater for the Δ10*t*,12*c*-18:2 treatment, which may correspond to the earlier findings showing that the Δ10*t*,12*c*-18:2 isomer had a reducing effect on AA. There was no dose response with cells treated with 10, 30 and 60 μM concentrations of Mix suggesting that the concentration

difference was not large enough to elicit a difference in effect on cell growth (Figures 6-2 and 6-3). Based on the phospholipid data it would be expected that the effect of the Δ10*t*,12*c*-18:2 isomer on levels of AA may potentially affect cell growth to a greater extent relative to the Δ9*c*,11*t*-18:2 isomer, which was not evident. One important consideration in this assay is that the incorporation of ^{3}H-thymidine is merely an estimate of cell growth within a narrow window of time, therefore effects of the Δ10*t*,12*c*-18:2 isomer may not have been captured.

The effect of CLA on membrane phospholipid composition and tumor cell growth is linked mechanistically by the action of eicosanoids. It is known that the MDA-MB-231 mammary tumor cell line produces the eicosanoid, PGE_2, which is associated with enhanced tumor growth [30; 42-44]. It has been shown previously that AA is released in mammary tumor cells by phospholipase A_2 [2; 45], or through mechanisms of phorbol ester activation of PKC and PLC [34]. In the present study, treatment with AA+LA(60) and AA+LA(120) enhanced PGE_2 production indicating that exogenous AA was converted to PGE_2 and also AA derived from LA conversion to AA (Figure 6-10). All 3 CLA treatments, Mix, Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2 inhibited PGE_2 synthesis relative to AA+(LA60) and AA+LA(120) treatments. It appears that both the Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 isomers equally inhibited PGE_2 synthesis. Based on the earlier phospholipid data the Δ10*t*,12*c*-18:2 isomer strongly reduces AA, thus it would be expected that cells treated with Δ10*t*,12*c*-18:2 would produce the least amount of PGE_2. This disparity is most likely due to differences in the designs of the separate experiments. The membrane phospholipid data was collected at 48 hrs in unstimulated cells (Tables 6-2, 6-3, 6-4 and 6-5) and PGE_2 was collected from stimulated cells after 24 hrs. Some amount of the Δ10*t*,12*c*-18:2 isomer was also present in all CLA mixtures. It is likely that the amount of Δ10*t*,12*c*-18:2 present in all the mixtures contributed to the reduction in PGE_2. It is unknown what changes in levels of PGE_2 occurred during the 24 hr incubation period and possibly levels of PGE_2 in each of the treatments may have already been maximally inhibited at differing time points. Another possibility is that the level of AA may not have declined sufficiently by 24 hrs to see a treatment effect due to Δ10*t*,12*c*-18:2, however prolonged stimulation after 24 hrs results in cell death. Inclusion of

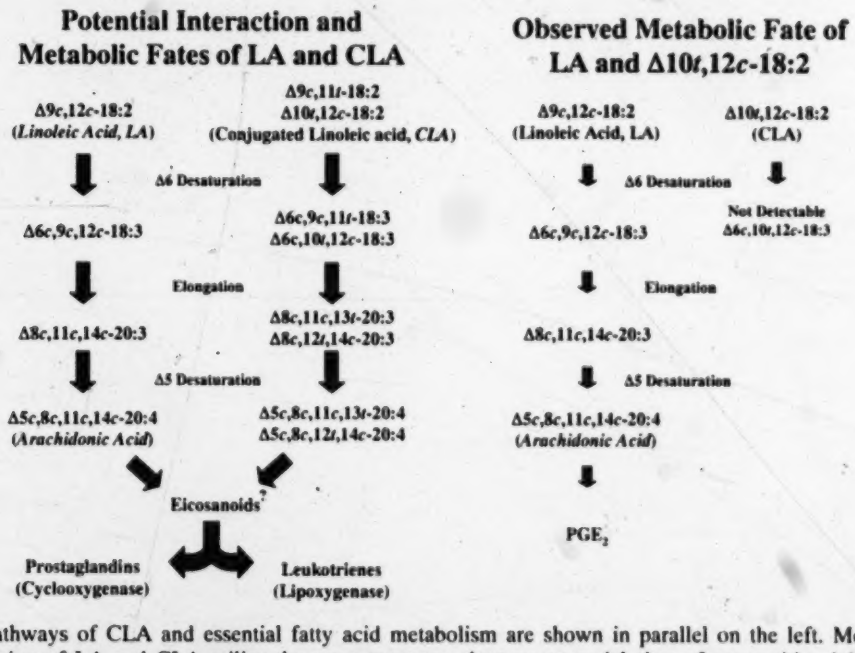
exogenous AA may have masked some effect of Δ10*t*,12*c*-18:2 on PGE_2 synthesis, thus reducing the sensitivity of the assay to detect treatment effects.

Inhibition of elongation and desaturation of LA to AA would suggest that this is the result of competitive inhibition by Δ10*t*,12*c*-18:2 for enzymes of the same pathway. Fatty acid analysis by GC-MS did not indicate that such products derived from Δ10*t*,12*c*-18:2 were produced. This is in contrast to previous studies indicating that both Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 are converted [8] to conjugated 18:3 and 20:4 products. Although this conversion does occur, only small amounts are produced [6]. CLA cultured in transformed yeast expressing fungal Δ6 desaturase did not produce any detectable conjugated 18:3 metabolites [46]. It was also observed that CLA inhibited the conversion of LA, to its desaturated metabolite, γ-linolenic acid (GLA, 18:3n6), and most strongly by Δ9*c*,11*t*-18:2. This effect is in contrast to this study showing that Δ10*t*,12*c*-18:2 was more potent. These differences may be due to the different cell systems employed. Overall, these observations in combination with the results of this study suggest that CLA is not readily metabolized and conversion of LA to AA is competitively inhibited by CLA.

No new eicosanoids were detected by GC-MS. It has been reported that the MDA-MB-231 cell line also produces leukotrienes of LTB_4, 12- and 15-HETE. None of these leukotrienes were detected. It is most likely that these cells did not produce sufficient quantities for detection with magnified losses due to extraction and derivatization. Both PGE_2 and LTB_4 spiked into culture media at a concentration of 1 μg / 15 mL (70 ng / mL) media were successfully extracted and detected. Recovery was not determined. Given that CLA was not elongated and desaturated, production of CLA derived eicosanoids would be unlikely. Elongated, desaturated products of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 have been identified as Δ5,8,11,13-20:4 and Δ5,8,12,14-20:4, respectively [8; 9]. The first step in the conversion of AA to all prostaglandins is the abstraction of the methylene-interrupted hydrogen at carbon 13 by cyclooxygenase, which is absent in these 20 carbon conjugated fatty acids [47]. Thus the synthesis of new prostaglandins from conjugated metabolites of CLA is highly unlikely. This is also true for 12- and 15-lipoxygenase which produce 12- and 15-HETEs respectively. It may be possible for 5-lipoxygenase to utilize these 20 carbon conjugated metabolites as it requires the

methylene interrupted hydrogen at carbon 7 which is a shared trait between AA and CLA metabolites. Determining if 5-lipoxygenase can act on these elongated, desaturated CLA metabolites was not possible in this study as there was an absence of any CLA metabolites.

Figure 6-11 Proposed and Metabolic Interaction Between LA and CLA



The pathways of CLA and essential fatty acid metabolism are shown in parallel on the left. Metabolic conversion of LA and CLA utilize the same enzymes, thus are potential sites of competitive inhibition. Shown as a "?" are potentially derived eicosanoids, which have yet to be determined. Shown on the right is the observed metabolic fate and interaction of LA and CLA based on the findings of the present study. The Δ10*t*,12*c*-18:2 isomer and not metabolites inhibit the conversion of LA to AA, resulting in reduced PGE_2 synthesis in the MDA-MB-231 mammary tumor cell line.

This study demonstrates that the mechanism of action by which CLA inhibits tumor growth is due to the inhibition of LA to AA conversion. This effect is strongly associated with the action of the Δ10*t*,12*c*-18:2 but not the Δ9*c*,11*t*-18:2. Reduction in AA provides a rationale for the reduced levels of PGE_2 and growth as estimated by ^{3}H-thymidine incorporation. There are many sites in the pathway of essential fatty acid metabolism, which can be affected via competition with CLA isomers. This study suggests that CLA mediates its effects at the first step of this pathway (Figure 6-11).

In the present study a mechanism of CLA action has been demonstated in a cell culture model. Future work is required to establish the biological significance of these findings in a larger animal model or in human studies. Future work may also include exploring the role of phospholipase A_2, a key step in substrate release, and the role of CLA in other types of cancers.

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Chapter 7 – Effect of Enriched CLA Mixtures on Leukotriene Synthesis by HL-60 Cells

1. Introduction

The results from Chapter 6 demonstrate that effects of CLA on essential fatty acid metabolism result in the modulation eicosanoids derived by cyclooxygenase synthesis. The other pathway of eicosanoid synthesis is mediated by the actions of lipoxygenases, which produce leukotrienes. Very little is known about the specific effects of the individual Δ9*c*,11*t*- and Δ10*t*,12c-18:2 isomers on leukotriene synthesis.

CLA has also been shown to reduce lipoxygenase products of splenic LTB_4 and lung LTC_4 in mice using a mixture of isomers [1]. The objective of the present study is to determine if enriched mixtures of CLA isomers can inhibit leukotriene synthesis in the HL-60 cell line.

Cells of the immune system such as neutrophils readily produce leukotriene products upon stimulation. HL-60 cells are often used as models of neutrophil function. HL-60 cells incubated in dimethylsulfoxide (DMSO) are induced to differentiate into neutrophil like cells, which produce 5-HETE and LTB_4 [2]. LTB_4 and 5-HETE are both produced by 5-lipoxygenase.

2. Materials and Methods

A. Maintenance of HL-60 Cells

HL-60 cells were obtained from the ATCC. Cells were seeded at a density of 3-5x10^6 cells in 75 cm^2 vented flasks in a total volume of 50 mL media containing FBS (5 or 10% v/v) which was added to RPMI Medium 1640 (Gibco BRL, Cat No. 31800-022, Grand Island, NY, USA) at the time of media replacement. Stock RPMI was supplemented with A/M (1% v/v) (Gibco BRL, Cat No. 15240-062, Grand Island, NY, USA) and buffered with 2.0 g $NaHCO_3$ and HEPES buffer solution (2.5% v/v, 25 mL/1000 mL media) (Gibco BRL, Cat No. 15630-080, Grand Island, NY, USA). The pH

was then adjusted to 7.4 before being sterile filtered. Cells were passaged every 2-4 days depending upon confluency and level of FBS used.

B. CLA Treatment and Collection of Media from HL-60 Cells

Cells were seeded at a density of $3x10^5$ cells/mL into 75 cm2 vented flasks containing 10% FBS in RPMI media. Cells were differentiated in DMSO (0.6 mL, 1.2% v/v) (Sigma, Cat No., D-2650, Oakville, Ontario, Canada) for 5 days. Cells were transferred to a 50 mL centrifuge tube and pelleted at 300 x g for 10 min. Fatty acid treatments were prepared as described in Chapter 6, section H and adjusted for a total volume of 2 mL. Cells were resuspended in phosphate buffered saline ($2-3x10^7$ cells/mL) with 1.4 mM calcium, pH 7.4 (2 mL) and transferred to the 15 mL centrifuge tubes. Cells were incubated for 15 min in a 37°C shaking water bath. Cells were stimulated with A23187 (10 μM) (Sigma, Cat No. C-7522, Oakville, Ontario, Canada). Stock A23187 was stored in DMSO at a concentration of 5 mg/mL at room temperature. The reaction was incubated for 10 min in a 37°C shaking water bath. The reaction was stopped with ice cold MeOH (2 mL). 12-Hydroxystearate (1 mg) was added as the internal standard. The pH was adjusted to pH 4 with formic acid (5 μL). The supernatant was diluted with ddH_2O (2 mL) and applied to a Sep Pak column (Waters Corporation, Cat No., WAT036935, Milford, Massachusetts, USA) that had been re-equilibrated with MeOH (1 mL), then PBS (1 mL, pH 4.0). Columns were washed with ddH_2O (1 mL), then dried. Leukotrienes were eluted with ethyl acetate (2 x 2 mL) (Fisher Scientific, Cat No. E145-4, Fair Lawn, NJ, USA). The leukotrienes were converted to their t-butyldimethylsilyl ether methyl esters as described in Chapter 6, section I.

C. GC-MS Analysis

GC-MS conditions are the same as those described in chapter 6, section B. The prominent diagnostic ions used to detect PGE_2, LTB_4, 5-HETE, 11 deoxy-PGE1 and 12-hydroxystearate are listed in Table 7-1. The presence of new eicosanoids were also monitored using m/z = 73 as the diagnostic ion.

Table 7-1 Prominent Ions of Eicosanoids and Standards Detected by GC-MS

Molecule	Prominent ions
LTB_4	467, 335, 245, 73
5-HETE	391, 245, 73
11-deoxy-PGE_1	438, 406, 374, 73
12-hydroxystearate	371, 339, 229, 75, 73

D. Statistical Analysis

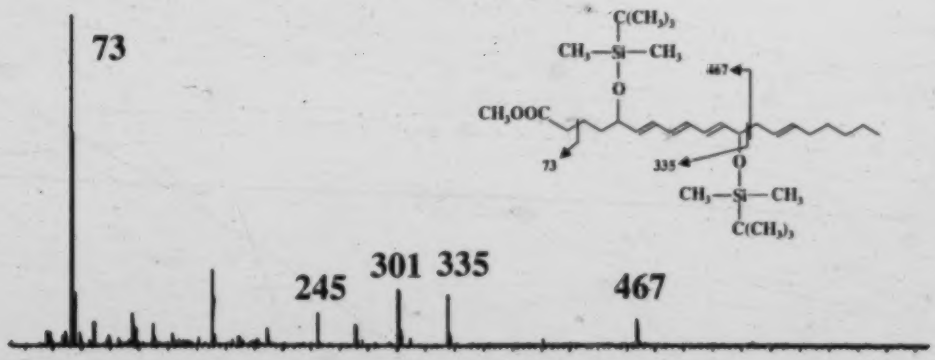
Replication in the experiment refers to the use of different passages (n=5). Effects of CLA treatments on LTB_4 and 5-HETE synthesis was analyzed by 1-way ANOVA blocked by passage number using the SAS version 8 statistical package. Duncans multiple range comparisons were performed for balanced data.

3. Results

A. Effect of CLA Mixtures on 5-HETE and LTB_4 Synthesis by HL-60 Cells

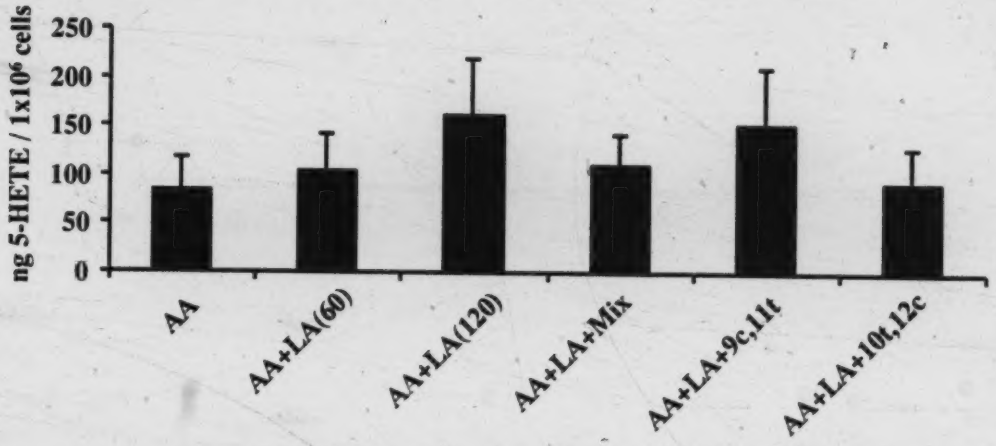
LTB_4 and 5-HETE were readily identified by their characteristic ions by GC-MS as well as 12-hydroxystearate, the internal standard. An ion scan of the LTB_4 standard is shown (Figure 7-1). There were no differences amongst all the treatments on 5-HETE and LTB_4 synthesis (Figures 7-2 and 7-3, respectively).

Figure 7-1 GC-MS Total Ion Chromatogram of LTB_4



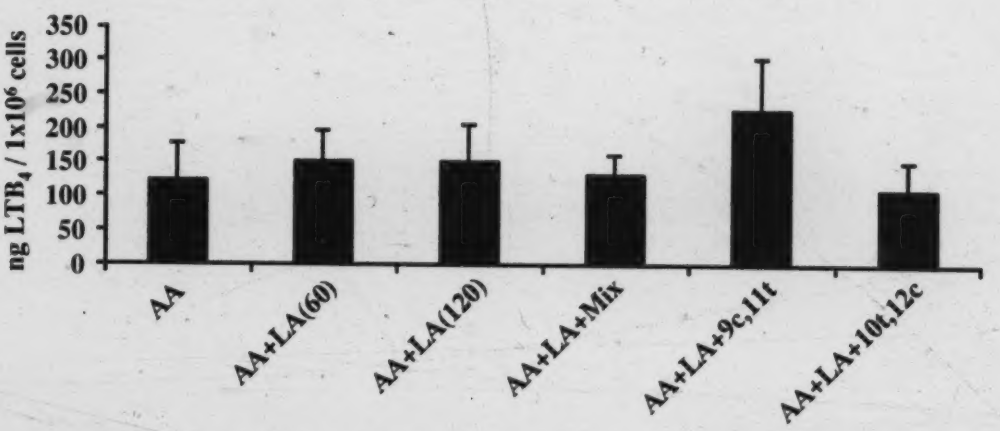
The GC-MS fragmentation pattern of LTB_4 and its derivatized structure is shown above.

Figure 7-2 Effect of CLA Mixtures on 5-HETE Synthesis by HL-60 Cells



Values are Mean ± SEM, n=5 for each treatment. There was no significant effect of treatment on the synthesis of 5-HETE by 1-way ANOVA, blocked by passage number. 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2. All fatty acids were provided at a concentration of 60 μM, unless otherwise indicated.

Figure 7-3 Effect of CLA Mixtures on LTB_4 Synthesis by HL-60 Cells



Values are Mean ± SEM, n=5 for each treatment. There was no significant effect of treatment on the synthesis of LTB_4 by 1-way ANOVA, blocked by passage number. 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2. All fatty acids were provided at a concentration of 60 μM, unless otherwise indicated.

4. Discussion

The objective of this study was to determine the specific effects of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 on the synthesis of leukotrienes. In this model system cells are stimulated for a short burst during which time, eicosanoids are rapidly produced. Assessment of substrate availability from membrane phospholipid can not be discerned from this model as in Chapter 6. Conclusions can only made about the effect of CLA on 5-lipoxygenase activity and/or competition for AA. Neutrophils have a requirement for exogenous AA and are unable to synthesize AA from LA [3], which further limits this experimental design to specifically assess affects of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 on lipoxygenase activity. The results from this study show that CLA does not inhibit even in the presence of AA leukotriene synthesis via the 5-lipoxygenase pathway. These results are similar to those reported by Truitt et al. [4]. Using isolated platelets stimulated in the presence of either Δ9*c*,11*t*- or Δ10*t*,12*c*-18:2 in the presence of AA, it was shown that both isomers directly inhibited cyclooxygenase but not lipoxygenase activity.

The present study demonstrates that CLA does not directly inhibit lipoxygenase activity by competing with AA. This indirectly indicates that CLA exerts its effects on eicosanoid synthesis at the level of substrate availability. This study also demonstrates that CLA does not modulate leukotriene synthesis in neutrophils, and suggests that CLA may not modulate this component of immune function.

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Chapter 8 – Summary Discussion and Future Direction

1. General Summary

A method to synthesize a simple mixture of CLA isomers from safflower oil is described. Safflower oil, rich in linoleic acid was extracted and alkali isomerized producing a mixture high in CLA consisting primarily of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 in equal amounts **(Objective 1a)**. The separation of CLA isomers was improved using a new highly polar GLC column, and the Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 were identified by partial hydrazine reduction **(Objective 1b)**. Starting from a mixture of CLA isomers containing predominantly Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 in equal amounts, a method was devised to enrich each of these isomers by counter-current urea crystallization **(Objective 1c)** These methods developed were used to analyze CLA content in Canadian dairy and beef products. Only the Δ9*c*,11*t*-18:2 isomer was detected and levels ranged between 1.2-6.2 mg/g fat. **(Objective 1d)**

The results obtained indicate that MDA-MB-231 cells supplemented with LA and CLA on an equal molar basis, preferentially incorporated LA into all membrane phospholipid fractions. Equal or enriched mixtures of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 initially incorporated into phospholipid fractions in the same proportion as the starting mixture. Over time, the level of Δ10*t*,12*c*-18:2 declined and Δ9*c*,11*t*-18:2 was preferentially retained. LA accumulated and AA was reduced in membrane phospholipid fractions when MDA-MB-231 cells were treated with an equal mixture of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 or an enriched mixture of Δ10*t*,12*c*-18:2 **(Objective 2a)**. All CLA treatments inhibited the proliferation of MDA-MB-231 cells **(Objective 2b).** No metabolites of CLA were detected, suggesting that MDA-MB-231 cells do not readily desaturate or elongate conjugated fatty acids **(Objective 2c).** All CLA treatments reduced PGE_2 synthesis by MDA-MB-231 cells **(Objective 2d)**. No LTB_4 was detected in culture media from MDA-MB-231 cells. All CLA treatments had no effect on LTB_4 or 5-HETE synthesis by HL-60 cells **(Objective 2e)**. No new eicosanoids were detected in the culture media from MDA-MB-231 or HL-60 cells **(Objective 2f).**

Hypothesis 1

It was hypothesized that CLA displaces LA and AA, precursors of eicosanoids, from membrane phospholipid, thus reducing the synthesis of the eicosanoids, PGE_2 and LTB_4. The results obtained show that CLA does not displace LA, but inhibits the conversion of LA to AA from membrane phospholipid fractions as a mechanism of reducing AA. CLA treatment corresponded with reduced PGE_2 synthesis and growth.

Hypothesis 2

It was hypothesized that CLA is elongated and desaturated to 20 carbon products such as Δ5*c*,8*c*,11*c*,13*t*-20:4 and Δ5*c*,8*c*,12*t*,14*c*-20:4 which compete with AA for cyclooxygenase and lipoxygenase thus reducing the synthesis of the eicosanoids, PGE_2 and LTB_4. The results obtained suggest that CLA is not readily desaturated and elongated. The absence of CLA metabolites suggest that they do not have a significant role in modulating essential fatty acid metabolism or eicosanoid synthesis.

Hypothesis 3

It was also hypothesized that these 20 carbon products metabolized via 5-lipoxygenase yields uncharacterized eicosanoids, predicted to be isomers of LTB_4, such as 14-hydroxy LTB_4 and 10-hydroxy LTB_4. The absence of 20 carbon metabolites of CLA prevents this hypothesis from being answered conclusively. The lack of substrate suggests that very little CLA is metabolized to 20 carbon elongated, desaturated metabolites and hence the formation of such hypothesized leukotrienes is not likely to occur in detectable amounts.

Hypothesis 4

The MDA-MB-231 cell line was a good model to assess the effect of CLA on prostaglandin synthesis. It was initially proposed that the effect of CLA on leukotrienes would also be assessed using this cell line. The MDA-MB-231 cell line has been shown previously by other investigators to produce the leukotrienes, LTB_4, 12- and 15-HETE, however these leukotrienes were not detected under the conditions used in the present

study. To assess the effect of CLA on the lipoxygenase pathway another cell line was selected. The neutrophil differentiated HL-60 cell line was selected on the basis that it readily produces the leukotrienes 5-HETE and LTB_4. CLA treatments did not have an effect on 5-HETE or LTB_4 synthesis by HL-60 cells.

2. Conclusions

This thesis expands and extends upon essential methodologies for approaching and conducting research on CLA. The development of these methodologies has provided a foundation upon which to instigate studies examining the anti-carcinogenic mechanism of CLA action. The data presented in this thesis identifies a mechanism of action and the specific isomer responsible. The main contributions of this thesis are as follows:

1. CLA can be synthesized from a vegetable oil rich in LA after urea purification. Isomerization of LA to CLA requires careful attention to the reaction conditions, which determines the complexity of the mixture produced.
2. Separation of CLA isomers is improved using the SP-2560 highly polar GLC column. Identification of the geometry and position of double bonds of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 were accomplished using partial hydrazine reduction.
3. Enriched mixtures of Δ9*c*,11*t*- or Δ10*t*,12*c*-18:2 can be produced via counter-current urea crystallization.
4. The Δ10*t*,12*c*-18:2 isomer inhibits the metabolism of LA to AA. An equal mixture of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 and an enriched mixture of Δ10*t*,12*c*-18:2, but not an enriched mixture of Δ9*c*,11*t*-18:2 reduced AA, and increased LA in membrane phospholipid fractions of the MDA-MB-231 mammary tumor cell line. Conversion of LA to AA in the presence of an enriched mixture of Δ9*c*,11*t*-18:2 was similar to cells treated with LA alone.
5. All CLA mixtures inhibited the production of cyclooxygenase derived PGE_2 from stimulated MDA-MB-231 mammary tumor cells, and also inhibited their growth as estimated by ^{3}H-thymidine incorporation.

6. None of the CLA mixtures inhibited the synthesis of 5-lipoxygenase derived LTB_4 and 5-HETE by neutrophil differentiated HL-60 cells.

3. Implications of Thesis

The conclusions from this thesis suggests a key role for the Δ10*t*,12*c*-18:2 CLA in inhibiting tumor growth. This is in contrast to previous work suggesting that the Δ9*c*,11*t*-18:2 isomer is the biologically active isomer [1-3]. The anti-carcinogenic effect of the Δ9*c*,11*t*-18:2 isomer has been based on the assumption that this isomer is found in significantly greater abundance in ruminant food products [4] relative to the Δ10*t*,12*c*-18:2 isomer. The greater biological abundance was assumed to have greater biological activity and relevance. Due to methodological limitations, CLA could only be conveniently synthesized and studied as a mixture of isomers also containing the Δ10*t*,12*c*-18:2 isomer. The biological relevance and contribution of these isomers could not be ascertained using these mixtures. The development of a method in this thesis to partially purify these two major isomers was the key element in collecting the data to demonstrate that the synthetic Δ10*t*,12*c*-18:2 isomer likely has greater anti-carcinogenic potency than the Δ9*c*,11*t*-18:2 isomer.

A clearer understanding of the mechanism of action is also demonstrated. The results show that the Δ10*t*,12*c*-18:2 isomer modulates essential fatty acid metabolism by inhibiting the metabolic conversion of LA to AA and is likely the isomer responsible for reducing the synthesis of the tumor growth promoting eicosanoid, PGE_2.

Breast cancer is the leading cause of mortality in Canadian women afflicted with cancer. It is estimated that in 2001, approximately 5500 women will die and some 19,500 new cases of breast cancer will be reported [5]. Conventional acute treatment of breast cancer through surgery and chemotherapy has been successful to a certain degree in treating this disease. It should not be forgotten that prevention is an equally advantageous strategy in combating this disease [6]. Preventative strategies such as diet intervention can utilize CLA to minimize long term risk and disease outcomes by inhibiting the formation, or the progression of this disease.

There is strong evidence indicating a link between diet fat and breast cancer. Animal and cell-culture investigations support a role of specific dietary fatty acids in the promotion and suppression of breast cancer. The most well studied types of fatty acids are the n-6 [7-9] and n-3 [9-11] series of fatty acids, which promote and suppress tumor development, respectively in animal models. CLA can also be added to this list of important modulatory fatty acids [12; 13].

Eicosanoids, especially PGE_2 are elevated in the most aggressive types of metastatic breast cancer [14]. Overproduction of PGE_2 is associated with primary breast cancers [15]. The exact role of PGE_2 in breast cancer is unknown but appear to aid in tumor growth, inhibition of apoptosis, metastasis and increased angiogenic potential [14-18]. The increased synthesis of PGE_2 serves a second function to suppress the immune system [19]. Reduction of PGE_2 by CLA may inhibit tumor growth and prevent the suppression of the host immune system.

4. Limitations

The key limitation of the work presented in this thesis is the model system used to investigate the effect of CLA on breast cancer. The chief argument is the relevance of the model to mimic exactly what would occur *in vivo*. Arguably, this is difficult to conclude with absolute certainty. The chief advantage of cell culture systems are their usefulness in elucidating mechanisms of action, used to explain phenomena already observed in animals and humans. Cell culture systems can also be used to show efficacy or a rationale prior to conducting further work in animals and humans. For these reasons, the *in vitro* work in this thesis provides a mechanism of action that explains previous findings in other cell culture and animal studies and it also provides the basis for future work to further confirm the present findings in animal and human studies.

5. Future Work

As mentioned in the previous section, animal and human studies are needed to reaffirm the mechanism of action shown in this thesis. The next step would be to show in

animal models that the Δ10*t*,12*c*-18:2 isomer is indeed more potent than the Δ9*c*,11*t*-18:2 isomer.

Both cyclooxygenase and lipoxygenase products are believed to be involved in aspects of breast cancer [9]. The synthesis of leukotrienes by mammary tumors is believed to play an autocrine role by inhibiting apoptosis of tumor cells [18]. Treatment of MDA-MB-231 mammary tumor cells with inhibitors to the cyclooxygenase and lipoyxgenase pathways inhibits proliferation [20; 21]. This suggests that in addition to prostaglandins, leukotrienes produced by cancerous cells also have important biological activity. The effect of CLA on leukotriene synthesis in cancerous cells is unknown. In this thesis, it has been demonstrated that CLA reduces prostaglandin synthesis by reducing substrate availability, therefore similar effects on leukotriene synthesis may also be expected. The study of CLA and its impact on cancer has largely focused on the breast cancer model, however, these results can be readily extended to the study of other forms of cancers with a component of upregulated eicosanoid metabolism.

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Appendix A – Summary of Biological Studies

Although not exhaustive, Tables A-1, A-2 and A-3 summarize and highlight the findings from a number of studies investigating the biological effects of CLA on cancer, atherosclerosis and nutrient metabolism.

Table A-1 Anticancer effects of CLA

Reference	Experimental Model	CLA/Diet Diet Composition	• Major Finding
[1-14]	General Reviews		
Anticancer effects of CLA: Animal Studies			
[15-17]	DMBA induced skin tumorigenesis, SENCAR or CD-1 mice	a) Organic solvent extract from beef preparations b) Control animals received acetone	• Preparation inhibited total numbers and incidence of mouse epidermal tumors either as natural or synthetic form.
[18]	DMBA induced skin tumorigenesis, CD-1 mice	Treatments applied topically a) Alkali isomerized linoleic acid b) HPLC purified beef extract c) Acetone or LA control	• Active ingredient in beef identified as CLA • Both incidence and number of tumors was reduced in synthetic CLA group, and not different between acetone control and LA
[19]	Benzo(a)pyrene (BP) induced tumorigenesis in forestomachs of female ICR rats	CLA mixture – given by gavage a) mL CLA+0.1 mL olive oil b) mL LA + 0.1 mL olive oil c) 0.1 mL olive oil	• CLA inhibitied mouse forestomach induced by benzo(a)pyrene • Only Δ9*c*,11*t*-18:2 incorporated into total phospholipid of forestomach
[20]	DMBA induced mammary tumorigenesis in Female Sprague-Dawley Rats	AIN-76A basal diet supplemented with 0.5, 1 or 1.5% CLA mixture by weight.	• CLA inhibited tumor incidence and cumulative tumor weight • Total mammary adenocarcinomas was reduced 32, 56 and 60% at 0.5, 1 and 1.5% CLA by weight, respectively • 1% CLA by weight provided maximal protection • Only Δ9*c*,11*t*-18:2 incorporated into total phospholipid in mammary and liver tissues
[21]	2-amino-3-methylimidazo[4,5-f]quinoline (IQ) DNA adduct forming carcinogen in CDF1 mice	CLA mixture given by gavage Tissues collected 24 hour post IQ. Control animals given trioctanoin	• CLA inhibited DNA adduct formation in some organ systems

[22]	DMBA or methylnitrosourea (MNU) induced mammary tumorigenesis in Female Sprague-Dawley Rats	CLA mixture provided at 0.05, 0.1, 0.25 or 0.5 by weight in diet. Diets fed for 5 weeks from weaning until carcinogen administration at 50 days of age	• 0.1% CLA is minimum requirement to reduce mammary tumor yield, which equates to 3 g CLA for 70 kg man • CLA inhibits tumors induced by either DMBA (requiring metabolic activation) and MNU (direct alkylating agent) • CLA provision during puberty (day 30-60 in rats) confers protection against mammary tumorigenesis and proliferative activity of lobuloalveolar structures in mammary gland is inhibited by CLA
[23]	MNU induced mammary tumorigenesis in female Sprague-Dawley rats	CLA mixture provided as free fatty acid or triglyceride preparation in AIN-76A diet at 1% by weight of diet. a) Diets provided from weaning until day 56 as either free fatty acid or triglyceride b) Free fatty acid diet fed from weaning until day 42 or 56. MNU given on either day 42 or 56. c) MNU administered at day 56, followed by diet administration for either 1,2 or 5 months post	• No difference between free fatty acid or triglyceride form of CLA • Early CLA consumption during post weaning and adolescent period conferred long lasting protection after carcinogen injection even though CLA was removed from the diet • CLA administration post carcinogen injection required continuous CLA intake to maintain protection. • CLA is effective in protecting the mammary gland when provided during prepubertal and pubertal periods of growth
[24]	IQ induced tumorigenesis in colon of male F344 rats	CLA mixture given by gavage on alternating days in weeks 1-4. IQ given by gavage every other day in weeks 3 & 4. Control received safflower oil.	• CLA had no effect on size of foci but inhibited number of aberrant crypt foci in colon • CLA inhibited IQ-DNA adduct formation in colon but not liver • CLA inhibits carcinogen activation versus direct interaction with the procarcinogen • CLA does not scavenge electrophiles nor activate phase I detoxification pathways
[25]	DMBA induced skin tumorigenesis in female Sencar mice	CLA mixture given at 0 (control), 0.5, 1 or 1.5% by weight of diet. 4 weeks post DMBA treatment, TPA or acetone applied twice weekly for 25 weeks.	• First appearance of tumors was delayed and tumor incidence was reduced with 1.5% CLA treatment • Tumor yield was inhibited with 1 and 1.5% CLA Tumor size was unaffected with CLA treatment • 35 weeks post TPA administration, tumor incidence and number remained constant

[26]	DMBA induced mammary tumorigenesis in female Sprague-Dawley Rats	Maintained on 20% corn oil diet, then intubated with 10 mg of DMBA at day 50. 1% CLA diet started 4 days post and maintained for either 4, 8 or 20 weeks.	• CLA withdrawn after 4 or 8 weeks of CLA treatment resulted in rapid increase in tumor incidence and was similar to controls receiving no CLA. Only continuous CLA treatment for 20 weeks reduced tumor incidence and burden • CLA had no effect on frequency or distribution of codon 61 mutant ras gene • Withdrawal of CLA results in depletion from neutral lipid to a greater extent than phospholipid. Neutral lipid maybe a better marker of protection than phospholipid
[27]	DMBA induced mammary tumorigenesis in female Sprague-Dawley Rats	CLA mixture used. Diets started 1 week before 7.5 mg DMBA given at day 50 a) 10, 13.3, 16.7 or 20% (by weight of diet) of vegetable fat blend comparable in composition to avg. US diet, with or without 1% CLA b) 20 or 8 % corn oil + lard, with or without 1% CLA c) 20% corn oil with 0.5, 1 or 1.5% CLA	• CLA inhibited tumor incidence and total number of tumors at a constant rate regardless of the level of fat. • Incorporation of CLA into phospholipid (0.3%) and neutral lipid (2-3%) was different in magnitude. No change in distribution of fatty acids in total phospholipid with CLA treatment • CLA in 20 or 8% corn oil diets inhibited incidence and total number of tumors regardless of the level of linoleate • At a constant level of 20% corn oil in diet, increasing levels of CLA inhibited tumorigenesis was maximal at 1% (similar to previous results using 5% corn oil [23]. • CLA was observed to inhibit lipid peroxidation in mammary tissue assessed by MDA levels
[28]	DMBA induced mammary tumorigenesis in female Sprague-Dawley Rats	CLA mixture used. Initially maintained on 2 or 12% LA diet. At 55 days of age, 10 mg of DMBA was administered. 4 days post, 0.5, 1, 1.5 or 2 % CLA was added	• Rats receiving 2% LA + CLA had fewer incidence and number of tumor bearing rats, suggesting that 2% LA is not maximal for tumor promotion in DMBA model. • 1% CLA conferred maximal protection expressed as % inhibition in either 2 or 12% LA treated groups • LA and AA were not displaced from neutral lipid or total phospholipid.

[29]	DMBA induced mammary tumorigenesis in female Sprague-Dawley Rats	1% CLA mixture added to AIN76-A diet. DMBA given at day 50. a) control, 0% CLA b) 1% CLA from weaning to 50 days of age c) 1% CLA from day 55 to termination d) 1% CLA from weaning to termination	• Neutral lipid and phospholipid content was not different between 1% CLA and control diet. Conjugated 18:3 and 20:3 fatty acids were identified in mammary tissue • Density of mammary epithelium was reduced 21% with CLA • Proliferation of terminal end buds and lobuloalveolar buds was decreased with CLA • CLA treatment at time of mammary gland maturation is critical period for protection against future development of tumors
[30]	Immunodeficient CB-17 scid/scid mice injected with MDA-MB468 breast adenocarcinoma	0 or 1% CLA mixture blended with commercial mouse food pellets. Diet given 2 wks prior to MDA-MB468 innoculation and diets continued to end of the study.	• Weight and volume of tumors inhibited with CLA at 9 or 14 wks post inoculation. • Histological examination revealed less organized and invasive growth of tumor cells into mammary ducts or rosettes and no metastasis, to the lungs, peripheral blood and bone marrow with CLA treatment
[31]	Female BALB/c mice injected with WAZ-2T (-SA) tumor line into inguinal mammary fat pad	Alkali isomerized safflower oil was source of CLA mixture. Basal diet contained 4.1% corn oil with 0.9% combination of CLA + safflower oil blended to achieve 0, 0.1, 0.3 or 0.9% CLA. Diets fed for 3 or 6 wks	• PHA induced lymphocyte proliferation increased with CLA at wk 3, but not wk 6. Con A or LPS lymphocyte stimulation was unaffected by CLA diet treatment. Lymphocyte cytotoxicity was unaffected by CLA. IL-2 production by splenic lymphocytes was enhanced with CLA • Volume, wt and latency of aggressive established tumor unaffected by CLA • Lipid peroxidation in mammary tissue unaffected by CLA
[32]	Immunodeficient CB-17 scid/scid mice, injected with DU-145 human prostatic adenocarcinoma	CLA mixture or LA mixed with commercial food pellets • 2 weeks after diets started, mice innoculated with DU-145 cells and maintained on diet for 14wks	• Tumor mass lowest in CLA group • 10, 80 & 100% of mice showed metastatic spread to lungs with CLA, LA and control group, respectively • ICAM-1, marker of tumor burden lowest in CLA group

[33]	MNU induced mammary tumorigenesis in female Sprague-Dawley Rats	CLA mixture added to AIN-76A diet.	• 1% CLA maximal for reducing terminal end bud branching • Inhibiting degree of branching corresponded to anticarcinogenic response • Fraction of CLA metabolized to conjugated 18:3 and 20:3 in mammary and liver in dose dependent manner • CLA did not displace LA but did displace 18:3, 20:3 and 20:4 in mammary but not liver
[34]	MNU induced mammary tumorigenesis in female Sprague-Dawley Rats	Macronutrient composition similar to humans a) control butter fat, 0.1% CLA b) high CLA butter fat, 0.8% CLA c) Matreya CLA + butter fat d) Nu Chek CLA + butter fat	• CLA reduced by 25% mammary epithelium, epithelium per unit area of fat pad and terminal end bud branching • Tumor incidence and yield decreased with CLA treatment regardless of source of CLA • Butter CLA resulted in higher accumulation in mammary ad peritoneal fad pad relative to Matreya and Nu Check CLA • Δ9*c*,11*t*-18:2 is the principle anticarcinogenic isomer. Δ9*c*,11*t*-18:2 accumulated in tissue most readily regardless of source and level present in the mixture (92, 81 and 25% in butter, Nu Check and Matreya, respectively)
[35]	12-O-tetradecanoylphorbol-13-acetate (TPA) induced skin tumorigenesis in female CD-1 or Sencar mice	CLA mixture added to AIN-76 diet at 0(control), 0.5, 1, 1.5% by weight of diet	• As CLA increased, 16:1 and LA decreased in neutral and total phospholipid • PGE_2 was decreased with 1.5% CLA diet • c-myc mRNA was reduced but not significantly by CLA • No effect of CLA on ODC – marker of tumor promotion • 1 and 1.5% CLA modestly inhibited hyperplasia
[36]	Balb/cAnN mice transplanted with mouse mammary tumor cell line, 4526	CLA mixture added to semipurified diet containing 20% corn oil at 0, 0.1, 0.5 and 1% weight of diet. Positive control group received indomethacin.	• 0.5% CLA was maximal for delaying latency of tumors but growth rate of tumors was similar among CLA treatments • Metastasis to lung decreased with CLA treatment and was similar to levels in indomethacin treated animals • Tumor burden (size, vol) was significantly reduced by CLA relative to indomethacin treated animals

Anticancer effects of CLA: Cell Culture Studies

[37]	Human M21-HPB malignant melanoma; HT-29 colorectal; and MCF-7 breast cancer cells	CLA mixture	• CLA inhibited proliferation of cancer cells
[38]	MCF-7 breast cancer cells	CLA mixture or LA provided at concentrations between 1.78 - 7.14×10^{-5}M in culture media. Cells incubated for 12 days with treatments	• LA is initially stimulatory and later inhibitory • CLA is inhibitory at all concentrations, in a dose and time-dependent manner • At times when LA and CLA were both inhibitory, CLA was more inhibitory relative to LA
[39]	Lung adenocarcinomas, A-427, SK-LU-1 and A549 and a human glioblastoma cell line, A172	Cells were supplemented for 3 days. a) CLA mixture or LA provided at 10, 20 or 40 μM in culture media. b) 40 μM CLA + Vitamin E (10, 20 or 30 μM) c) 40 μM LA + Vitamin E (10, 20 or 30 μM)	• CLA inhibited lung carcinoma proliferation in a dose and time dependent manner • LA generally had no effect on proliferation • Vitamin E partially restored cell growth in the presence of CLA, suggesting that CLA promotes formation of cytotoxic lipid peroxidation products. Partial restoration also indicates that CLA has effects independent of lipid peroxidation
[40]	MCF-7 mammary tumor cell line and HMEC (human mammary epithelial cells, normal control)	LA or CLA mixture cultured in media	• LA stimulated proliferation and CLA was inhibitory • LA increased lipid peroxidation but not CLA
[41]	MCF-7 (estrogen responsive) and MDA-MB-231 (estrogen unresponsive) mammary tumor cell lines	LA and CLA mixture co-cultured in media	• Proliferation of estrogen responsive MCF-7 was inhibited by CLA to a greater extent relative to MDA-MB-231 • C-myc expression in MCF-7 was down regulated by CLA
[42]	MCF-7 mammary tumor cell line and SW480 colonic tumor cell line	CLA mixture added to culture media at 0 (control), 5, 10 and 20 ppm for 4,8 & 12 days in ethanol	• Dose dependent inhibition of growth • CLA inhibited ^{3}H-leucine, ^{3}H-uridine, and ^{3}H-thymidine incorporation into protein, RNA and DNA, respectively • Lipid peroxidation increased as measured by TBARS for MDA in dose dependent manner • CLA acts as prooxidant and activates SOD, catalase and glutathione.

[43]	MCF-7 mammary tumor cell line	LA or 98-99% Δ9*c*,11*t*-18:2 added to culture media in ethanol complexed to BSA at 0, 0.18, 0.36 or 1.78×10^{-5}M	• LA stimulatory and CLA inhibitory to cell growth • Providing 0.18×10^{-5}M CLA did not change total saturated, mono-unsaturated and polyunsaturated fatty acid composition • No effect of CLA on phospholipase C or protein kinase C • No effect of CLA or LA on PGE_2 production

Table A-2 Anti-atherogenic effects of CLA

[44]	General Review		
[45]	New Zealand White rabbits, fed cholesterolemic diet	0.5 g CLA/day/rabbit mixture + semi-synthetic diet containing 0.1% cholesterol by weight in diet	• No difference in overall weight, plasma triglyceride, LDL-c • CLA tended to reduce effect of cholesterol, but not significantly • CLA improved LDL/HDL ratio • CLA showed less fatty streak lesion, but not significantly • Histological exam of aortas and connective tissue showed less evidence of atherogenesis
[46]	Male Hamsters, fed cholesterolemic diet	Commercial non-purified ration + 10 % coconut, 1 % safflower, 0.12 % cholesterol + 0.06, 0.11, or 1.1 %energy CLA mixture and 1.1% LA	• CLA decreased plasma total cholesterol, non HDL-c, plasma triglyceride • No change in body weight and no difference in HDL-c • CLA decreased aortic fatty streak area • No difference in α-tocopherol levels
[47]	Male F_1B hybrid hamsters, fed cholesterolemic diet	CLA mixture, enriched Δ9*c*,11*t*-, or Δ10*t*,12*c*-18:2 incorporated into diets containing 0.1 g/kg cholesterol in purified diet. Controls received no CLA	• Δ10*t*,12*c*-18:2 isomer exerted effects similar to the CLA mixture, reducing LDL-c and HDL-c • Δ9*c*,11*t*-18:2 in general had no effect on lipid parameters assessed
[48]	Female C57BL/6 mice, fed cholesterolemic diet	a) 5 or 2.5 g CLA/kg b) 5 g LA/kg for controls c) 10 g/kg cholesterol diets d) fed for 15 wks	• No difference in serum cholesterol or HDL-c • 5 g CLA/kg had increased HDL-c/total cholesterol ratio • CLA decreased triglyceride • CLA increased aortic fatty streak formation
[49]	Male Sprague-Dawley rats, perfused liver model of lipid transport	1% LA or CLA added to AIN 76 diet	• CLA fed animals produced more ketone bodies and decreased cholesterol secretion from liver • CLA lowered triglyceride but not significantly

[50]	Male Golden Syrian Hamsters, fed mildly cholesterolemic diet	a) Nu Chek mixture of CLA 10 g/kg, Δ9*c*,11*t*-18:2 Matreya 2g/kg or LA 2g/kg b) All diets had equal amounts of Δ9*c*,11*t*-18:2 and 0.05 g/100g cholesterol in diet	• CLA mixture decreased weight gain • CLA mixture, but not Δ9*c*,11*t*-18:2 decreased plasma triglyceride and total cholesterol • No effect on HDL-c
[51]	Male New Zealand White rabbits, fed cholesterolemic diet	CLA mixture added to a semipurified diet containing 0.1 or 0.2% cholesterol	• 0.1% CLA inhibited Atherosclerosis by 34% • 1% CLA regresses established atherosclerosis
[52]	Male Sprague-Dawley rats, fed non enriched cholesterol diet	Semi-synthetic cholesterol free diet, based on AIN 93 with either safflower oil (control) or 1, 3 or 5% CLA mixture	• CLA reduced serum cholesterol primarily in LDL-c • No change in triglyceride in serum and lipoprotein
[53]	Albino rats fed non-enriched cholesterol diet	CLA mixture added to semi-purified diets at 0.5, 1.0 or 1.5% of diet	• 1% CLA increased body weight gain and feed conversion, but not at 0.5 or 1.5% • HDL-c/total cholesterol ratio significantly and triglyceride significantly increased with CLA • CLA reduced LA and AA in abdominal fat pads
[54]	HepG2 cell line, assessment of single fatty acid effects	LA, Δ9c,11c-, 10*t*,12*c*-18:2 or 16:0 in 1%	• Δ10*t*,12*c*-18:2 but not Δ9c,11c-18:2 inhibited apoB secretion • 10*t*,12*c*-18:2 inhibits triglyceride synthesis • both CLA isomers inhibit cholesterol ester synthesis

Table A-3 Studies Reporting Effects of CLA on Whole Body Metabolism

Reference	Study Parameters	Isomer Composition	Major Finding
[10; 55; 55; 56; 56]	General Review		
Studies Reporting Effects of CLA on Whole Body Metabolism: Body Compositional Studies			
[57]	Primigravid female Fisher Rats, model of rat development and growth when CLA is provided at different stages in life	CLA mixture provided at 0, 0.25, and 0.5 g/100g of diet mixed in corn oil and added to a non-purified diet	• Pups receiving CLA during gestation and lactation were significantly heavier than controls • Fuel efficiency – weight gain/g of food intake increased in dose dependent manner • No difference in food intake among animals

[58]	ICR mice, model of body compositional effects. 3T3-L1 adipocyte cell line	CLA mixture added to semi-purified diet Fed for 32 days a) 5.5% corn oil (control) b) 5% corn oil + 0.5% CLA by weight of diet	• No effect of diet on body weight or feed intake • CLA reduced body fat while protein (absolute and relative) and body water was increased • CLA increase β-oxidation (measured by carnitine palmitoyl transferase) in fat pad and skeletal muscle but not in liver • CLA added in culture to 3T3-L1 inhibited adipocyte LPL activity and increased free glycerol in culture media indicating an increase in lipolysis
[59]	Pig model of body composition	Either 2% sunflower or 2% CLA provided in diet.	• CLA decreased feed intake and improved feed conversion efficiency and no change in average daily weight gain • CLA effects not gender specific • CLA increased lean and decreased subcutaneous fat
[60]	Male inbred AkR/J mice, model of body compositional change	• High (45%) or low fat (11%) with or without CLA mixture (2.46 mg/kg diet) • 6 week feeding trial	• CLA significantly reduced body weight and energy intake • CLA reduced body fat and also carcass protein • CLA independent of diet (high or low fat) increased energy expenditure
[61]	Female pigs, growing finisher pigs	Fed CLA mixture at 0, 0.7, 1.4, 2.75, 4.1 and 5.5 g/kg of diet	• No effect on average daily gain or feed intake • CLA increased gain to feed ratio by 6.3% • Dose dependent decrease in P_2 back fat thickness • CLA decreased fat to lean ratio with increasing CLA
[62]	ICR Female mice, model of body composition change during different times of CLA exposure	a) 0.5% CLA + 5% corn oil b) 5.5% corn oil control	• No change in body weight but feed intake reduced • Absolute decrease in whole body fat, but not water, protein and body water • Relative changes in body fat and whole body water • Effects of CLA when provided from weaning then withdrawn after 4 weeks persisted, including lower body fat, higher body water, and protein • Clearance of Δ10*t*,12*c*-18:2 was more rapid than Δ9*c*,11*t*-18:2 when CLA was withdrawn from diet
[63]	Male inbred AKR/J mice, model of body composition change	CLA mixture added to either high or low fat diet (45 or 15% kcal fat)	• CLA reduced body weight in either high or low fat diet groups • Adipose weight was reduced, but liver and spleen weights increased • CLA increased energy expenditure

[64]	ICR Female mice, model of body composition change with different CLA mixtures	a) 0.5% CLA mixture b) 0.3% enriched Δ9*c*,11*t*-18:2 c) 0.25% enriched Δ10*t*,12*c*-18:2 d) 0.5% CLA mixture – obtained from Nu Chek e) 0.9% dehydrated castor oil • Adipocyte 3T3-L1 cell line	• Only enriched Δ10*t*,12*c*-18:2 had significantly reduced body weight • Body composition analysis showed similar effects of the mixture and enriched Δ10*t*,12*c*-18:2 to reduce body fat, LPL activity, and increase protein, whole body water and ash while Δ9*c*,11*t*-18:2 had no effect on any of these parameters • Inhibitory effect of Δ10*t*,12*c*-18:2 was dose-dependent
[65]	Adult female women	17 women given 3 g CLA mixture/day for 64 days in a metabolic suite	• No change in fat-free mass, fat mass, % body fat, body weight • No change in energy expenditure
Studies Reporting Effects of CLA on Whole Body Metabolism: Energy Utilization Studies			
[66]	Pigs, adult sows fed isoenergetic diet	0 or 1% CLA mixture by weight of diet 6 week feeding trial	• No effect of CLA on digestibility and metabolizability of food • No effect of CLA on nitrogen balance • No change in weight of animals
[67]	Adult female pigs, fed isoenergetic diets. Pig metabolism more similar to humans than rodents.	0 or 1% CLA by weight in diet	• no change in body weight • CLA increased serum insulin • No effect of CLA on T4, T3 and β-hydroxybutyrate, α-tocopherol, protein, glucose, urea, creatinine and blood ATP • CLA tended to decrease blood leukocyte counts but not red blood cell counts • Serum triglyceride, cholesterol and phosphpolipid increased in LDL and VLDL but no changes in HDL • LDL/HDL ratio increased
[68]	German Landrace finisher pigs, model of energy metabolism feeding isoenergetic diets in a growing animal	CLA mixture added to provide 0 or 3% CLA by weight in diet	• No marked change in energy balance • No change in fat deposition, but slightly greater N retention with CLA

Studies Reporting Effects of CLA on Whole Body Metabolism: Effect on Adipose Tissue

[69]	Murine 3T3-L1 adipocyte cell line	CLA mixture added in DMSO to culture at 25-100 μM	• Inhibited differentiation in dose-dependent manner • PPAR γ2, C/EBPα, and aP2 mRNA were inhibited • CLA inhibited proliferation only in pre-confluent cells • Glycerol-3-phosphate dehydrogenase activity was inhibited
[70]	Murine 3T3-L1 adipocyte cell line	CLA mixture added to culture in ethanol at 0, 0.5, 1.0, 5.0, or 10 mg/mL concentrations	• CLA did not affect cell viability but did reduce cell proliferation of preadipocytes • CLA increased glucose uptake, de novo lipogenesis, fatty acid concentration and lipid droplet size
[71]	Sprague-Dawley rat model of white and brown adipose tissue response to CLA	CLA mixture added to AIN 93G diet a) 7% safflower oil (control) b) 6% safflower + 1% CLA c) 5% safflower + 2% CLA	• No difference in food intake, weight gain, tissue weights of white and brown adipose tissue, and liver • CLA decreased triglyceride and free fatty acid in white adipose tissue but not in brown adipose tissue

Studies Reporting Effects of CLA on Whole Body Metabolism: Effect on Diabetes

[72]	Male Zucker diabetic fatty rats, and lean litter mates and CV-1 cells	3 isocaloric diets containing a) 5% corn oil + 1.5% lard (control) b) 5% corn oil + 1.5% CLA mixture c) 5% corn oil + 1.5% CLA + 0.2% troglitazone (positive control, TZD) d) lean litter mates on control diet	• Food intake same, but CLA fed groups had significant lower final body weight relative to TZD group • CLA and TZD reduced insulin, but still higher than lean litter mates and TZD was more potent than CLA • CLA and TZD lowered circulating FFA, returned glucose to baseline after glucose tolerance test • CLA induced PPARγ activation in dose dependent manner in CV-1 cells • CLA and TZD increased aP2 mRNA expression in adipose tissue (marker for fat cell differentiation)

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Appendix B – Fatty Acid Composition of Fetal Bovine and Human Serum

1. Objective

To determine if the contribution of LA and CLA from FBS are significant relative to the level of these fatty acids added exogenously in experiments. The level of these fatty acids in human serum is also determined for comparison.

Table B-1 Fatty Acid Content of Fetal Bovine and Human Serum (μM)

	Fetal Bovine (unfiltered)	Fetal Bovine (filtered)	Human (unfiltered)	Human (filtered)
C12:0	4.3	0.2	0.4	0.04
C13:0	0	0	0	0
C14:0	1.5	1.5	3.0	1.1
C15:0	0.5	0.6	0.6	0.2
C16:0	13.6	13.3	79.6	55.1
C16:1 (n7) trans	0.1	0.6	0.1	0.3
C16:1 (n7) cis	2.0	1.6	8.2	4.8
C17:0	0.5	0.5	0.8	0.6
C18:0	5.6	5.8	26.7	17.9
C18:1 (n9) trans	0.1	0	0	0.04
C18:1 (n9) cis	10.1	11.8	74.2	43.6
C18:1 (n7) cis	2.9	2.5	4.7	2.7
C18:2 (n6)	3.0	4.4	83.9	25.6
C18:3 (n6)	0.1	0.2	1.0	0.4
C18:3 (n3)	0.1	0.1	1.0	0.2
CLA Δ9*c*,11*t*-18:2	0.2	0.1	0.8	0.2
CLA Δ10*t*,12*c*-18:2	0.01	0	0	0.02
CLA trans/trans	0.02	0	0	0.1
C20:3 (n6)	0.6	0.5	1.6	0.3
C20:3 (n3)	0	0	0	0.1
C20:4 (n6)	2.9	3.0	13.2	0.5
C22:1	0	0.1	0.2	0.2
C20:5 (n3)	0.2	0.1	0.04	0
C24:0	0	0.04	0.2	0.1
C22:4 (n6)	0.02	0.1	0	0.02
C22:5 (n6)	0	0	0.1	0.1
C22:5 (n3)	0.4	0.3	1.3	0.8
C22:6 (n3)	0.8	0.8	1.3	0.3

Values are adjusted to reflect the concentration present in culture media containing 5% serum (v/v).

2. Materials and Methods

The same lot of FBS was used for all experiments (n=1) (Gibco BRL, NY, USA, Lot No 1016785). Human serum was purchased (Cat No. H-1388, Sigma, Oakeville, Ontario, Canada). Lipids from FBS were extracted by the method of Folch [1]. Nonadecanoate methyl ester was added as an internal standard. Total lipid extracts were saponified in KOH-MeOH (0.5 M) and then methylated in boron triflouride (14% w/v). Fatty acid methyl esters were analyzed by GLC. Serum was analyzed from aliquots that were either unfiltered or filtered through a 0.22 μM membrane.

3. Results and Discussion

LA and CLA are typically used at a concentration of 60 μM in cell culture experiments. LA and CLA are both present in very low amounts in FBS (filtered or unfiltered, Table B-1) and therefore can be assumed to not contribute significantly to effects resulting from LA and CLA treatments.

4. Reference List

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Appendix C – Requirement for BSA to Complex Lipid in Cell Culture

1. Objective

The necessity to complex fatty acids to BSA prior to addition to culture media was assessed. Direct addition of fatty acids may be cytotoxic [1].

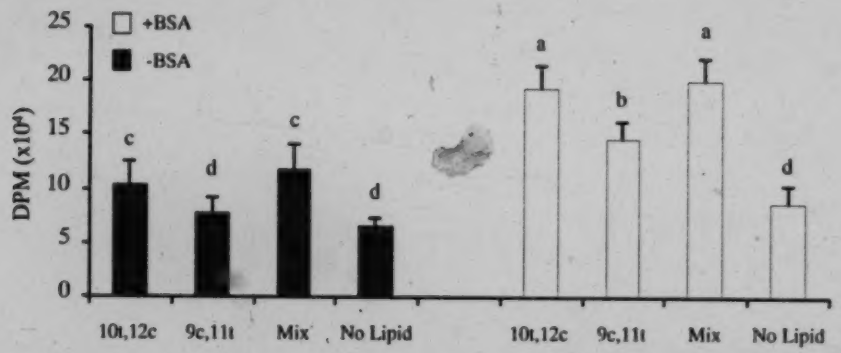
2. Materials and Methods

MDA-MB-231 cells were seeded at a density of 20,000 per well into sterile 24 well plates (Corning Inc., Corning, NY, USA). Cells were cultured in 1 mL IMDM supplemented with FBS (5% v/v) and A/M (1% v/v) for 2 days. Media was then replaced with IMDM supplemented with FBS (1% v/v), A/M (1% v/v) and one of the following lipid treatments, no lipid (control), Mix (60 μM), Δ9*c*,11*t*-18:2 (60 μM) or Δ10*t*,12*c*-18:2 (60 μM). All treatments were duplicated with the addition of BSA (0.1% w/v). Fatty acids were complexed to BSA by incubating in a shaking water bath for 30 min at 37°C. Cells were incubated in the fatty acid supplemented media for an additional 2 days. On day 4, cells were pulsed with tritiated thymidine (50 μL, 5 μCi, 85Ci/mmol) and incubated for 4 hrs. Cells were harvested and dried overnight before counting for radioactivity.

3. Results

The effect of the lipid treatments and BSA were analyzed by 2-way ANOVA. Both lipid treatment and BSA were significantly different ($p<0.01$). There was also a significant interaction effect ($p<0.01$). BSA significantly increased proliferation. Addition of lipid increased proliferation relative to the control, no lipid. Among the CLA treatments, proliferation was the lowest with Δ9*c*,11*t*-18:2 treatment. The interaction term (BSA x treatment) showed that treatment with lipids incubated in the presence of BSA enhanced proliferation between similar lipid treatments (Figure C-1).

Figure C-1 Effect of BSA and Different CLA Mixtures on the Proliferation of MDA-MB-231 Mammary Tumor Cells



Growth was assessed by the incorporation of radioactive ^{3}H-thymidine expressed in decays per minute (DPM). 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2. Treatment effects were analyzed by ANOVA and comparisons were done using the Pdiff function for unbalanced data. Values are mean ± SEM (n = 3-6 for each treatment). Different letters are significantly different ($p<0.05$).

4. Discussion

MDA-MB-231 mammary tumor cells cultured in media supplemented with fatty acids proliferate better when fatty acids are complexed to BSA. Direct addition of fatty acids into culture media reduced proliferation and may be cytotoxic to these cells under these conditions. The presence of BSA enhanced proliferation in parallel to the effects observed in the absence of BSA (Figure C-1). The direct cytotoxic effect of the fatty acids are not desirable and artificially reduce the effect of the lipid treatment. Therefore, lipids added in culture should be complexed with BSA prior to addition into culture media. CLA enhanced cell proliferation relative to cells treated only with media containing 1% FBS. This suggests that the addition of any exogenous fatty acids under these conditions enhances growth.

5. Reference List

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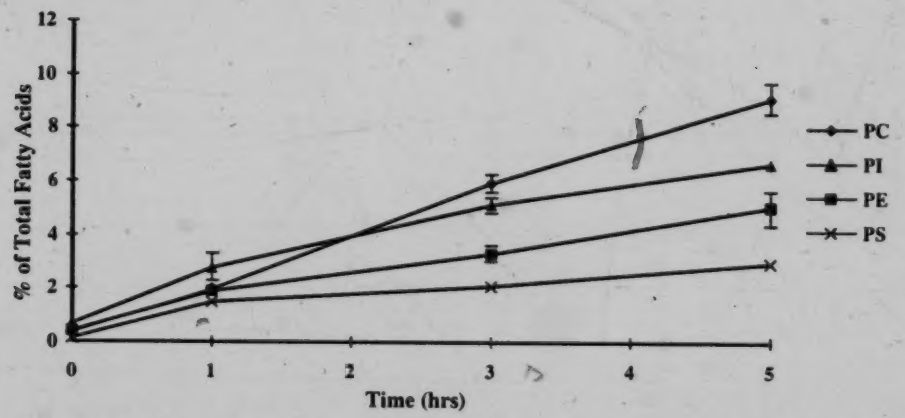
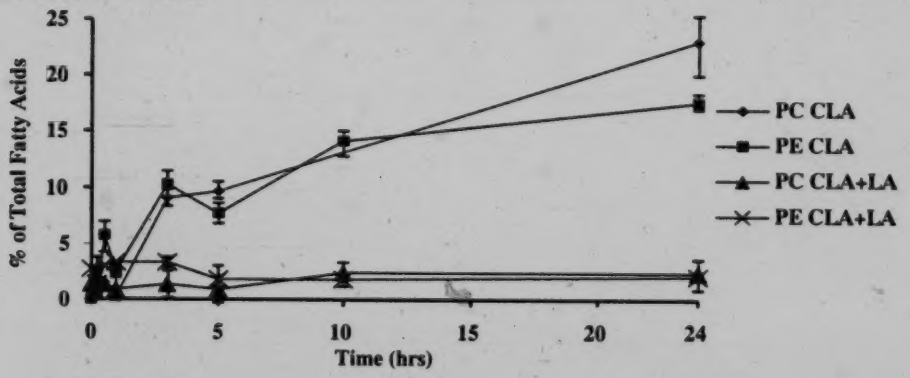
Appendix D – Preliminary Results Assessing the Incorporation of CLA in MDA-MB-231 Cell Membrane Phospholipid

Figure D-1 Incorporation of CLA into MDA-MB-231 Cell Membrane Phospholipid in the Absence of LA



A mixture of CLA isomers containing equal amounts of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 was provided in culture media (50 μM, 1% FBS). At 0, 1, 3 and 5 hrs cells were collected and lipids were extracted and separated into phospholipid classes by TLC and then methylated for FAME analysis by GLC. CLA preferentially incorporates into PC. Two flasks from the same passage were used for each time point (n=1).

Figure D-2 Incorporation of CLA into MDA-MB-231 Cell Membrane Phospholipid in the Presence of LA



A mixture of CLA isomers containing equal amounts of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 was provided in culture media (50 μM, 1% FBS) At 0, 5, 10 and 24 hrs cells were collected and lipids were extracted and separated into phospholipid classes by TLC and then methylated for FAME analysis by GLC. CLA incorporation is reduced in the presence of LA into PC, PS, PI and PE (PI and PS not shown). Two flasks from the same passage were used for each time point (n=1).

Appendix E – Fatty Acid Binding to BSA

1. Objective

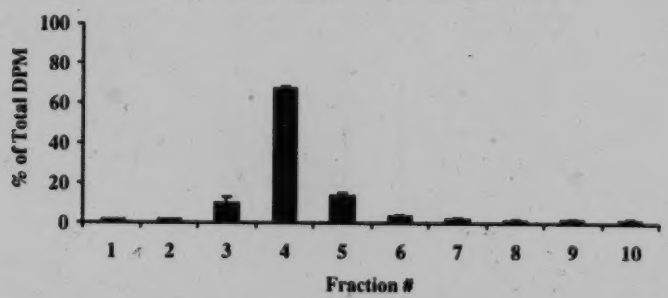
To verify the protocol used to complex fatty acids to BSA.

2. Materials and Methods

IMDM (9.5 mL) supplemented with A/M (1% v/v), BSA (0.1 g, 0.1% w/v), FBS (0.5 mL, 5% v/v) was incubated at 37°C for 30 min in a shaking water bath. A 2 mL aliquot was passed through a glass column (~40 x 1 cm) packed with Sephadex G-25 (coarse) (Pharmacia Fine Chemicals, AB, Uppsala, Sweden) in IMDM (1% v/v A/M). Fractions (10 x 2 mL) were collected. An aliquot (100 µL) was analyzed by the method of Lowry [1] for protein determination to identify the fractions containing BSA.

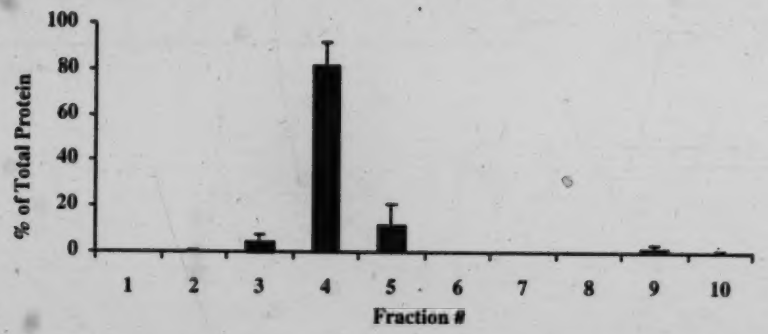
This above procedure was repeated with media containing 60 µM of LA consisting of ^{14}C-18:2 and cold 18:2. LA (33.1 µL, 5 mg/mL in EtOH) was dried down under nitrogen and low temperature in a sterile 15 mL centrifuge tube, then ^{14}C-18:2 (5 µL, 0.05 mCi/ 0.5 mL ; 53 mCi/mmol) (NEN, Cat No. NEC-501, Missassauga, ON, Canada) was added followed by IMDM (9.5 mL) supplemented with A/M (1% v/v), BSA (0.01 g, 0.1% w/v), and FBS (0.5 mL, 5% v/v). After eluting and collecting fractions from the Sephadex G-25 packed column, aliquots (2 x 100 µL) from each fraction were transferred to scintillation vials containing Scintisafe™ Econo1 (5 mL) (Fisher Scientific, New Jersey USA). Samples were then counted for radioactivity using a Beckman 5000 Liquid Scintillation Spectrometer and Counter (Beckman Instruments, Palo Alto, CA).

Figure E-1 Fractional Elution of ^{14}C-18:2 Complexed to BSA



Values are mean ± SEM from 4 determinations. Fractions 3-5 contained 91% of total radioactivity.

Figure E-2 Fractional Elution of BSA Determined by Lowry



Values are mean ± SEM from 3 determinations. Fractions 3-5 contained 98% of total recovered protein.

3. Results and Discussion

The greatest proportion of radioactivity from ^{14}C-18:2 eluted in fractions 3-5 which constitute 91% of total radioactivity (Figure E-1). Protein determination showed that BSA eluted between fractions 3-5 and contained 98% of total protein (Figure E-2). Elution of both radioactivity from ^{14}C-18:2 and protein within the same fractions confirm that LA was complexed to BSA.

4. Reference List

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Appendix F – Effect of LA on MDA-MB-231 Mammary Tumor Cell Growth

1. Objective

One hypothesis being tested is the growth inhibitory effect of CLA on cancerous cells. Therefore it is necessary to confirm that LA indeed enhances the growth of MDA-MB-231 mammary tumor cells.

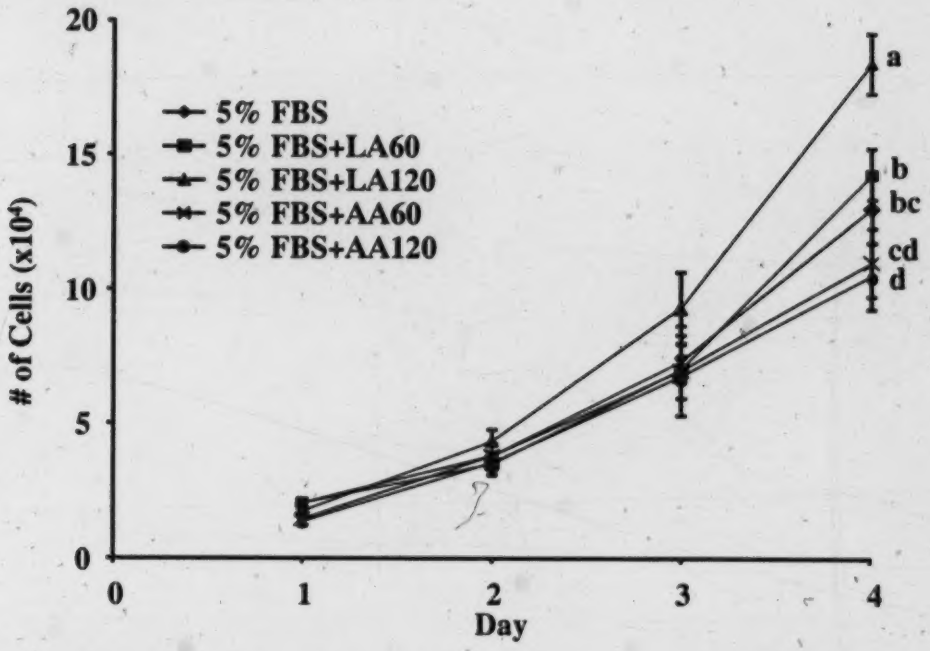
2. Materials and Methods

MDA-MB-231 cells were seeded at a density of 20,000 cells/well in 24 well sterile plates. Fatty acids were supplemented into culture media at a concentration of 60 or 120 μM. Fatty acid treatments were prepared similarly as described in Appendix E, containing BSA (0.1% w/v) and FBS (5% v/v) in IMDM. Each treatment was applied to 2 wells for each time point from a single passage of cells and replicated twice more. Wells were counted in duplicate using a hemocytometer and viability was assessed by trypan blue exclusion. Cells were incubated in 1 mL of fatty acid supplemented media for 4 days. Cells were harvested and counted each day.

3. Results and Discussion

Cell growth was significantly increased with increasing concentrations of LA in media containing 5% (v/v) FBS. By day 3, cell growth was significantly enhanced only by treatment with 120 μM LA. On day 4, both LA(60) and LA(120) treatments enhanced growth relative to all other treatments (Figure F-1). AA supplemented into culture media at 60 and 120 μM reduced cell numbers by day 4.

Figure F-1 Cell Growth of MDA-MB-231 Mammary Tumor Cells in Media Supplemented with LA or AA



Values are mean ± SEM (n=3 for each treatment). Treatments at day 4 were analyzed by one-way ANOVA and means are compared by the Duncans Multiple Range test. Treatments with different letters are significantly different ($p<0.05$).

Appendix G – Growth of MDA-MB-231 Mammary Tumor Cells Different Serum

1. Objective

To determine optimal growth conditions for MDA-MB-231 cells using either bovine or human serum.

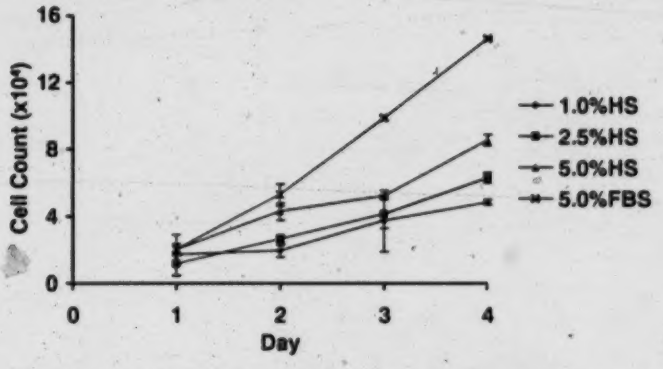
2. Materials and Methods

MDA-MB-231 cells were seeded at a density of 20,000 cells into 24 well flat bottom plates. Cells were cultured in either 1, 2, or 5% human serum (HS) or 5% FBS. Each treatment was applied to 2 wells for each time point from a single passage of cells. These treatments were replicated with another passage of cells. Wells were counted in duplicate using a hemocytometer and viability was assessed by trypan blue exclusion. Cells were harvested and counted each day.

3. Results and Discussion

Increasing levels of human serum supplemented into the culture media enhanced cell growth (Figure G-1). At the same level of supplementation 5% FBS enhanced growth to a greater extent than human serum (HS).

Figure G-1 Growth of MDA-MB-231 Cells in Human and Fetal Bovine Serum



Values are means ± SEM (n =2 for each treatment). HS – human serum and FBS – fetal bovine serum.

Appendix H – Stimulation of PGE_2 Synthesis by Calcium Ionophore, A23187

1. Objective

To assess the effect of different CLA mixtures on PGE_2 synthesis by MBA-MB-231 cells stimulated by the calcium ionophore, A23187.

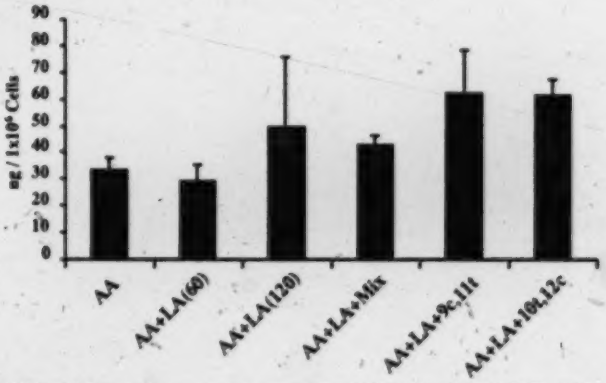
2. Materials and Methods

Cell culture conditions and fatty acid treatments are described in Chapter 6. Treatments were replicated using separate passages of cells. Cells were stimulated with A23187 (10 μM) for 1 day. Media was collected and 11-deoxy-PGE_1 was added to the culture media as an internal standard. The media was semi-purified using Sep Pak cartridges preconditioned with MeOH (2 mL) and ddH_2O (2 mL). The column was dried and then PGE_2 was eluted with ethyl acetate (2 x 5 mL). PGE_2 was then derivatized for GC-MS analysis as described in Chapter 6.

3. Results and Discussion

Co-stimulation by both the calcium ionophore and phorbol ester stimulated PGE_2 synthesis (Chapter 6). There was no significant stimulation of PGE_2 synthesis by calcium ionophore alone (Figure H-1).

Figure H-1 Effect of CLA Mixtures on PGE_2 Synthesis by MDA-MB-231 Cells Stimulated with A23187



Values are mean ± SEM (n=4 for each treatment, except n=3 for AA+LA+1012). There were no significant differences due to treatment effects.

Appendix I – Preliminary Results Assessing the Effect of CLA on PGE_2 and LTB_4 Production by MDA-MB-231 Mammary Tumor Cells by Enzyme Immunoassay

1. Objective

To determine if enriched mixtures of CLA isomers inhibit PGE_2 and LTB_4 synthesis in MDA-MB-231 cells using an enzyme immunoassay.

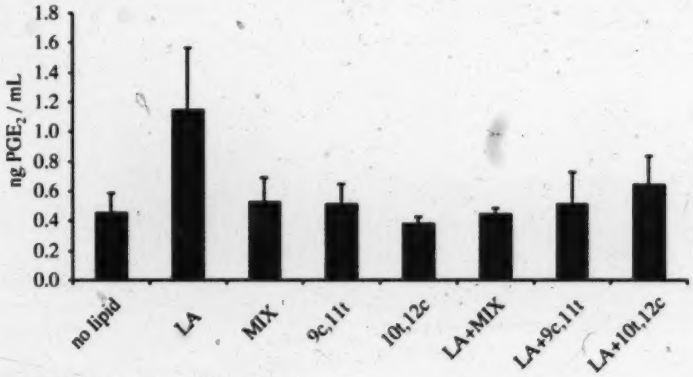
2. Materials and Methods

Assays were performed as indicated by the manufacturer (Cedar Lane Laboratories Limited, Oxford Biochemical Research Inc., Catalog No. EA 35 (LTB_4) and Catalog No. EA 02 (PGE_2), Hornby, Ontario, Canada). Replications are from 2 separate passages of cells.

3. Results and Discussion

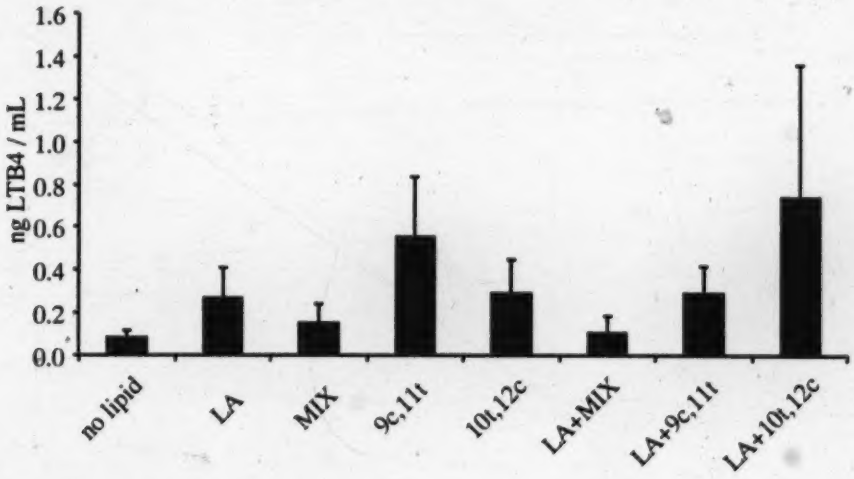
The results indicated that CLA inhibited PGE_2 synthesis but not LTB_4 (Figures I-1 and I-2). In this set of experiments LA used was of lower purity (< 90%). The experiment was replicated with LA (> 90%) (Figure I-3). The results were contrary to those in the first experiment and CLA enhanced PGE_2 and LTB_4 synthesis. The contradictory results suggest that there may be lot variability in the detection of PGE_2 and LTB_4.

Figure I-1 Effect of CLA Mixtures on PGE_2 Synthesis by MDA-MB-231 Cells



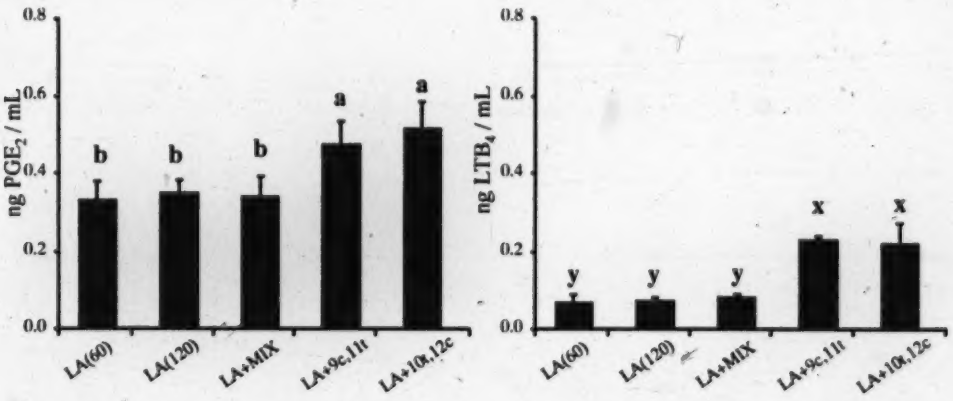
Values are mean ± SEM (n=2 for each treatment).

Figure I-2 Effect of CLA Mixtures on LTB_4 Synthesis by MDA-MB-231 Cells



Values are mean ± SEM (n=2 for each treatment).

Figure I-3 Effect of CLA Mixtures on PGE_2 and LTB_4 Synthesis by MDA-MB-231 Cells



Means ± SEM (n=4). Treatments with different letters are significantly different by Duncans multiple range test ($p<0.05$).

Appendix J – Fatty Acid Composition of MDA-MB-231 Cells Treated with Different Mixtures of CLA Over 48 hrs

Table J-1 Fatty Acid Composition in PC of MDA-MB-231 Cells Over 48hrs

	LA+Mix			LA+9c,11t			LA+10t,12c		
Fatty Acid	5hrs	24hrs	48hrs	5hrs	24hrs	48hrs	5hrs	24hrs	48hrs
C 16:0	20.5±1.3	24.3±2.4	27.1±1.1	20.8±1.3[b]	27.5±1.8[a]	26.9±0.9[a]	20.9±1.3	31.8±4.1	26.6±0.9
C 17:0	1.00±0.14	0.71±0.04	0.72±0.06	0.84±0.04	1.06±0.15	0.77±0.02	0.73±0.05	0.55±0.28	0.81±0.04
C 18:0	12.6±0.8[b]	11.3±1.4[b]	15.8±1.0[a]	13.1±0.3[b]	11.7±1.0[b]	15.8±1.1[a]	12.7±0.5	10.2±1.8	16.0±1.2
C 16:1(7)	1.12±0.22	0.94±0.20	1.08±0.13	1.67±0.42	1.40±0.20	0.85±0.37	1.17±0.20	1.11±0.23	1.29±0.20
C 18:1(9)t	1.76±0.52	0.80±0.31	1.22±0.19	0.95±0.18	1.32±0.46	1.12±0.05	1.39±0.34	0.75±0.30	1.16±0.17
C 18:1(9)	26.2±2.4	19.6±4.9	23.4±3.4	27.7±0.8	22.3±3.9	26.6±4.4	25.8±1.7	17.8±4.6	25.3±2.7
C 18:1(7)	5.93±0.35	4.81±0.46	4.84±0.28	5.80±0.31	5.62±0.61	4.28±0.37	6.01±0.37	4.11±0.63	4.82±0.20
C 24:1(9)	0.04±0.04	0.07±0.04	0.11±0.04	0.13±0.07	0.40±0.28	0.11±0.02	0.13±0.08	ND	0.41±0.30
C 18:2(6)	13.1±0.2	17.6±5.6	12.1±2.8	13.3±1.0	14.8±4.3	9.9±2.8	15.1±1.2	20.7±4.6	11.4±1.8
C 18:3(6)	0.21±0.14	0.11±0.05	0.23±0.03	0.31±0.06	0.20±0.10	0.25±0.03	0.12±0.06	0.10±0.05	0.31±0.07
C 20:2(6)	ND	2.08±0.91	1.52±0.36	0.72±0.01	1.47±0.36	1.15±0.45	0.79±0.06	1.20±0.71	1.33±0.26
C 20:3(6)	1.82±0.33	0.87±0.22	1.06±0.06	1.36±0.06	1.58±0.23	1.58±0.31	1.27±0.30	0.63±0.33	1.45±0.21
C 20:4(6)	4.90±0.22[a]	3.09±0.68[b]	2.70±0.05[b]	5.29±0.72	4.47±0.35	3.45±0.46	4.68±0.60	3.83±1.02	3.46±0.45
C 22:4(6)	0.92±0.03	0.99±0.13	0.99±0.14	1.07±0.12	1.23±0.15	1.14±0.10	0.86±0.08	1.19±0.40	0.85±0.44
C 22:5(6)	0.39±0.07	0.31±0.06	0.14±0.01	0.58±0.30	0.69±0.50	0.14±0.01	0.37±0.04	0.16±0.10	0.11±0.02
C 18:3(3)	0.32±0.23	0.08±0.04	0.09±0.03	0.19±0.12	0.69±0.68	0.09±0.05	0.21±0.16	0.16±0.15	0.08±0.04
C 20:5(3)	0.66±0.30	0.29±0.03	0.25±0.09	0.42±0.03[a]	0.18±0.09[b]	0.25±0.02[b]	0.25±0.13	0.18±0.09	0.22±0.06
C 22:5(3)	1.20±0.10	0.85±0.16	0.81±0.01	1.35±0.16[a]	0.88±0.05[b]	0.87±0.04[b]	1.17±0.14	0.97±0.28	0.85±0.09
C 22:6(3)	1.51±0.14	1.05±0.20	0.94±0.03	1.71±0.21[a]	1.14±0.17[b]	1.07±0.07[b]	1.58±0.20	1.35±0.49	1.11±0.09
CLA Δ9c,11t	2.75±0.82	5.61±1.92	2.58±0.85	1.63±0.55	0.70±0.40	1.99±1.40	1.20±0.10	1.00±0.61	0.78±0.41
CLA Δ11c,13t	ND	0.12±0.12	0.13±0.13	0.05±0.05	0.18±0.11	0.03±0.03	ND	0.16±0.16	0.59±0.41
CLA Δ10t,12c	2.12±1.05	4.06±2.21	1.94±0.65	0.69±0.57	0.07±0.05	1.06±0.78	2.95±0.52	1.79±1.28	0.83±0.45
CLA cis/cis	0.94±0.80	0.09±0.05	0.22±0.12	0.28±0.18	0.36±0.19	0.18±0.09	ND	0.03±0.03	0.19±0.19
CLA trans/trans	0.08±0.05	0.29±0.11	0.11±0.02	0.09±0.05	0.09±0.05	0.44±0.34	0.66±0.34	0.25±0.18	0.08±0.01

Values are mean ± SEM (n=3 for each time point for each treatment). Within each row of each treatment, treatments having different letters are significantly different ($p<0.05$). 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2 ; ND – not detected. All fatty acids were provided at a concentration of 60 μM, unless otherwise indicated.

Table J-2 Fatty Acid Composition in PE of MDA-MB-231 Cells Over 48hrs

	LA+Mix			LA+9c,11t			LA+10t,12c		
Fatty Acid	5hrs	24hrs	48hrs	5hrs	24hrs	48hrs	5hrs	24hrs	48hrs
C 16:0	4.29±0.58	7.26±2.83	5.25±0.23	4.56±0.66	5.90±1.48	4.86±0.25	4.74±0.45	4.89±0.50	5.12±0.28
C 17:0	0.33±0.18	0.60±0.06	0.40±0.07	0.25±0.13	0.31±0.16	0.43±0.07	0.34±0.34	0.49±0.10	0.44±0.07
C 18:0	24.8±0.6	24.4±1.0	24.7±0.2	25.8±0.7	26.6±1.9	24.3±0.3	25.6±0.6	25.4±0.1	24.6±0.5
C 16:1(7)	1.08±0.71	0.27±0.14	0.47±0.04	0.76±0.49	0.29±0.15	0.49±0.12	0.13±0.13	0.32±0.17	0.56±0.06
C 18:1(9)t	ND	0.27±0.20	0.26±0.13	ND	0.36±0.18	0.23±0.23	0.26±0.26	0.19±0.19	0.46±0.23
C 18:1(9)	24.9±0.8	19.1±3.3	21.4±3.8	29.0±2.0^a	20.2±1.3^b	23.3±3.2^b	27.2±1.5^a	19.5±1.7^b	22.5±3.2^b
C 18:1(7)	2.26±1.18	2.56±0.13	2.21±1.11	ND	2.52±1.26	2.26±0.89	0.09±0.09	3.66±0.45	1.80±0.66
C 18:2(6)	2.83±0.55^b	8.43±1.76^a	8.62±1.88^a	2.77±0.35^b	6.59±0.90^a	7.10±1.96^a	3.24±0.53^b	7.07±0.57^a	8.23±1.22^a
C 18:3(6)	ND	ND	ND	ND	ND	ND	ND	ND	0.02±0.02
C 20:3(6)	ND	0.90±0.45	1.44±0.15	ND	1.34±0.67	0.97±0.52	ND	0.82±0.42	1.80±0.13
C 20:4(6)	17.7±0.4	13.9±2.1	14.7±0.4	15.8±3.6	17.1±1.2	17.0±1.8	18.0±1.0	15.5±0.6	16.3±0.4
C 22:4(6)	4.71±0.15	5.15±0.31	5.63±0.60	4.48±0.33	6.38±0.50	5.74±0.17	4.37±0.32^b	6.16±0.64^a	5.90±0.61^a
C 22:5(6)	0.76±0.38	0.39±0.39	0.40±0.24	0.92±0.26	1.43±0.89	0.33±0.33	0.90±0.52	1.17±0.59	0.49±0.02
C 20:5(3)	1.45±0.17	0.52±0.28	0.75±0.05	2.15±1.36	0.50±0.29	0.48±0.24	0.69±0.36	0.97±0.12	0.59±0.19
C 22:5(3)	5.55 0.41	3.96 0.41	3.98 0.13	4.91 0.81	4.42 0.12	4.23 0.39	4.92 0.04	4.95 0.24	4.21 0.03
C 22:6(3)	8.65 0.30	5.80 0.59	5.92 0.52	8.41 0.11	5.76 0.38	5.91 0.31	9.15 0.96	6.33 0.38	5.54 0.10
CLA Δ9c,11t	0.33±0.33	3.12±1.06	2.10±0.71	0.12±0.12	0.25±0.14	1.45±1.12	0^b ±	0.56±0.15^a	0.69±0.14^a
CLA Δ11c,13t	ND	ND	ND	ND	ND	ND	ND	0.17±0.17	ND
CLA Δ10t,12c	0.34±0.34	3.35±2.10	1.79±0.62	ND	ND	0.89±0.89	0.44±0.44	1.67±1.00	0.72±0.40
CLA cis/cis	ND	ND	ND	ND	ND	ND	ND	0.20±0.20	0.06±0.06
CLA trans/trans	ND	ND	ND	ND	ND	ND	ND	ND	ND

Values are mean ± SEM (n=3 for each time point for each treatment). Within each row of each treatment, treatments having different letters are significantly different ($p<0.05$). 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ,; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2 ; ND – not detected. All fatty acids were provided at a concentration of 60 μM, unless otherwise indicated.

Table J-3 Fatty Acid Composition in PI of MDA-MB-231 Cells Over 48hrs

	LA+Mix			LA+9c,11t			LA+10t,12c		
Fatty Acid	5hrs	24hrs	48hrs	5hrs	24hrs	48hrs	5hrs	24hrs	48hrs
C 16:0	3.81±1.25	6.75±2.86	5.72±1.14	5.97±0.92	5.74±1.73	5.24±0.57	9.81±6.95	4.08±0.92	5.49±1.43
C 17:0	0.57±0.11	0.10±0.10	0.49±0.10	0.45±0.24	0.19±0.10	0.38±0.05	0.31±0.31	0.10±0.10	0.29±0.02
C 18:0	31.8±9.4	41.2±1.6	43.8±1.5	37.7±5.4	38.6±1.9	45.9±2.3	33.4±6.1	43.6±2.0	39.7±3.2
C 16:1(7)	0.16±0.08	0.06±0.06	0.16±0.08	0.14±0.07	ND	0.19±0.09	0.25±0.25	0.09±0.09	ND
C 18:1(9)t	0.83±0.20	ND	0.27±0.14	1.07±0.50	0.86±0.25	0.54±0.28	1.59±0.80	0.31±0.31	ND
C 18:1(9)	13.2±1.4	8.9±2.4	12.5±1.9	10.3±1.1	13.7±2.4	12.9±2.2	18.4±7.5	9.6±1.6	13.9±2.5
C 18:1(7)	3.77±0.38	2.93±0.71	3.90±0.56	3.16±0.44	3.36±0.26	3.68±0.55	3.68±0.41	3.35±0.43	4.39±0.94
C 24:1(9)	0.82±0.08[a]	0[b]	0[b]	0.63±0.48	ND	ND	ND	ND	ND
C 18:2(6)	9.79±1.99	9.24±3.17	6.52±1.45	6.85±1.41	6.17±1.54	4.46±1.40	6.58±2.34	9.49±2.50	5.88±0.67
C 18:3(6)	0.51±0.36	ND	0.06±0.06	0.08±0.08	ND	ND	ND	ND	ND
C 20:2(6)	0.00±0.00	0.68±0.68	0.88±0.88	ND	ND	ND	ND	ND	ND
C 20:3(6)	1.80±0.96	1.98±1.33	4.34±0.04	1.60±0.91	4.60±1.11	4.39±0.67	1.73±0.87	2.99±0.26	5.21±0.23
C 20:4(6)	20.3±3.0	13.4±2.0	14.6±0.6	16.2±1.7	19.1±2.9	15.2±1.1	14.3±5.1	18.4±0.5	17.6±1.2
C 22:4(6)	2.21±0.49	2.32±0.25	2.35±0.49	1.71±0.28	2.96±0.51	2.79±0.13	1.84±0.64	2.57±0.08	3.18±0.24
C 22:5(6)	3.09 0.91	3.23 1.90	0.65 0.34	4.95 2.95	2.47 1.44	0.90 0.22	2.20 0.57	1.21 0.44	1.40 1.08
C 18:3(3)	0.23±0.23	ND	ND	0.40±0.34	ND	ND	ND	ND	ND
C 20:5(3)	ND	ND	0.06±0.06	ND	ND	ND	0.14±0.14	0.07±0.07	ND
C 22:5(3)	1.17 0.19	5.39 4.27	1.25 0.23	6.67 5.56	1.14 0.09	1.67 0.45	2.28 0.43	1.52 0.11	1.41 0.19
C 22:6(3)	0.71 0.05	1.01 0.22	0.68 0.34	0.67 0.06	0.89 0.20	0.95 0.13	1.00 0.48	1.11 0.16	1.40 0.15
CLA Δ9*c*,11*t*	1.20±0.51	1.33±0.88	0.74±0.43	0.48±0.36	0.25±0.25	0.40±0.40	0.22±0.11	0.18±0.18	ND
CLA Δ11*c*,13*t*	ND	ND	0.04±0.04	ND	ND	ND	ND	ND	ND
CLA Δ10*t*,12*c*	2.71±1.35	1.42±1.11	0.89±0.45	0.61±0.53	ND	0.41±0.41	2.01±0.84	1.21±0.57	0.26±0.26
CLA cis/cis	0.26±0.14	ND	0.02±0.02	0.13±0.07	0.03±0.03	ND	0.04±0.04	ND	ND
CLA trans/trans	1.09±0.37[a]	0.08±0.08[b]	0.07±0.07[b]	0.25±0.20	ND	ND	0.23±0.23	0.12±0.12	ND

Values are mean ± SEM (n=3 for each time point for each treatment). Within each row of each treatment, treatments having different letters are significantly different ($p<0.05$). 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2 ; ND – not detected. All fatty acids were provided at a concentration of 60 μM, unless otherwise indicated.

Table J-4 Fatty Acid Composition in PS of MDA-MB-231 Cells Over 48hrs

	LA+Mix			LA+9c,11t			LA+10t,12c		
Fatty Acid	5hrs	24hrs	48hrs	5hrs	24hrs	48hrs	5hrs	24hrs	48hrs
C 16:0	5.65±0.23	6.34±0.63	4.99±0.53	8.04±2.89	6.23±1.21	4.96±0.66	4.05±0.63	6.34±0.56	5.94±1.34
C 17:0	1.00±0.05^a	0.85±0.09^a	0.51±0.01^b	0.66±0.11	0.27±0.27	0.52±0.10	0.87±0.18	0.73±0.04	0.53±0.02
C 18:0	40.6±1.9	37.1±5.8	47.2±1.0	45.9±1.8	39.6±1.2	48.6±1.7	39.2±0.7^c	44.0±0.5^b	46.3±0.1^a
C 16:1(7)	0.49±0.30	0.07±0.04	0.38±0.05	0.20±0.20	0.07±0.05	0.39±0.10	0.09±0.06	0.28±0.14	0.39±0.10
C 18:1(9)t	0.51±0.51	1.86±0.77	0.72±0.15	1.14±0.58	2.48±1.06	0.54±0.54	2.26±2.26	0.78±0.40	0.73±0.37
C 18:1(9)	35.3±2.6	27.4±6.7	20.2±1.9	29.6±1.5	24.2±7.5	23.4±5.3	35.2±2.0^a	19.8±1.7^b	22.1±1.8^b
C 18:1(7)	1.50±0.13^b	1.40±0.04^b	3.36±0.28^a	1.14±1.14	0.60±0.30	3.09±1.24	1.06±0.26^c	4.03±0.25^a	3.24±0.16^b
C 18:2(6)	3.03±0.03	8.84±1.90	6.45±1.41	1.46±0.80	5.45±0.95	3.94±1.52	3.17±0.08	7.26±1.58	5.88±1.07
C 18:3(6)	ND	ND	ND	ND	ND	ND	0.26±0.26	ND	ND
C 20:2(6)	ND	ND	0.28±0.28	ND	ND	ND	ND	ND	ND
C 20:3(6)	0^b	0^b	1.78±0.05^a	0.38±0.38	ND	0.98±0.52	0^b	1.83±0.20^a	1.83±0.25^a
C 20:4(6)	2.14±0.29	1.67±0.23	1.62±0.05	1.83±0.13	2.02±0.34	1.72±0.28	2.57±0.37	1.73±0.19	1.89±0.19
C 22:4(6)	2.02±0.20	2.16±0.24	3.27±0.15	2.62±0.29^b	1.62±0.35^b	3.98±0.17^a	2.16±0.13	2.77±0.36	3.62±0.34
C 22:5(6)	2.22±0.43	1.69±0.75	0.35±0.22	0.99±0.50	13.13±7.77	1.22±0.63	2.44±0.51	2.29±0.98	0.73±0.09
C 18:3(3)	ND	ND	0.04±0.04	ND	ND	ND	ND	ND	ND
C 20:5(3)	ND	ND	ND	0.05±0.05	ND	ND	ND	ND	ND
C 22:5(3)	2.09±0.09	2.70±0.70	2.80±0.68	2.80±0.48	1.59±0.54	2.48±0.38	2.46±0.29	3.44±0.32	2.87±0.34
C 22:6(3)	2.58±0.16	2.40±0.31	2.90±0.07	3.28±0.32	2.31±0.87	2.82±0.40	3.02±0.85	3.46±0.85	3.65±0.62
CLA Δ9*c*,11*t*	0.32±0.23	2.70±1.06	1.67±0.45	ND	0.09±0.07	0.83±0.83	0.23±0.12	0.25±0.25	ND
CLA Δ11*c*,13*t*	0.03±0.03	ND	ND	ND	0.11±0.07	ND	0.01±0.01	ND	ND
CLA Δ10*t*,12*c*	0.33±0.29	2.66±1.53	1.50±0.43	ND	ND	0.51±0.51	0.24±0.24	0.96±0.75	0.33±0.33
CLA cis/cis	0.27±0.24	0.18±0.18	ND	ND	0.17±0.17	ND	0.58±0.39	ND	ND
CLA trans/trans	ND	ND	ND	ND	0.03±0.03	ND	0.15±0.08	ND	ND

Values are mean ± SEM (n=3 for each time point for each treatment). Within each row of each treatment, treatments having different letters are significantly different ($p<0.05$). 9c,11t – Enriched mixture of Δ9*c*,11*t*-18:2 ; 10t12c – Enriched mixture of Δ10*t*,12*c*-18:2 ; Mix – equal amount of Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2 ; ND – not detected. All fatty acids were provided at a concentration of 60 μM, unless otherwise indicated.